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PHYSIOLOGICAL ZOÖLOGY

Volume XIX OCTOBER 1946 Number 4

FISSION AND DISINTEGRATION IN STYLARIA FOSSULARIS AT DIFFERENT TEMPERATURES

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THE distribution of the fission zone in *Stylaria fossularis* was studied by Chu and Pai (1945). According to their data, the location of the fission zone along the body axis is rather variable, ranging generally from the eighth to the twenty-fourth trunk segment; and if the number of dividing worms was plotted against the trunk segment at which fission takes place, a typical binomial curve could be obtained with its peak at the eighteenth trunk segment. From the data in that paper and a later one of Chu (1946) on piece regeneration, considerable light has been thrown upon the possible correlation between the phenomenon of natural fission and that of regeneration. Furthermore, the meaning of the wide-ranged variation in the location of the fission zone in natural populations has also been somewhat clarified through the genealogical analysis of fission within a small population established in the laboratory (Chu, 1945).

Following the line of these studies, we have continued the analysis along some other angles, i.e., the temperature effect on fission-zone distribution; the variation of the level of the meeting point of two disintegration gradients in *S. fossularis* following the application of toxic substances; and the possible effect of temperature on this variation.

SEASONAL VARIATION OF FISSION FREQUENCY

The method adopted to express the fission frequency in *S. fossularis* has been previously given (Chu and Pai, 1945). In order to detect the effect of temperature on fission, we made observations on three lots of two thousand worms in three different periods. In the first period we worked in July, 1944, and completed the observations within 17 days. The temperature varied in this period from 21°.7 to 29°.4 C. The results are shown in the first row of Table 1 and graph *A* of Figure 1. The location of the fission zone was found to range from the seventh through the twenty-third trunk segment, with highest frequency at the fifteenth trunk segment.

The second series of observations was carried out in November of the same year. We finished the analysis within 21 days. The variation of temperature was from 10°.2 to 17°.3 C. The distribution of the fission zone has now extended to a

TABLE 1

THE VARIATION OF FISSION FREQUENCY IN *Stylaria fossularis* AT DIFFERENT TEMPERATURES

	Fission Level (in Number of Trunk Segments Anterior to Fission Zone)																						Total Number of Worms Examined	Empirical Peak	Mean	Temperature Range (° C.)	Average Temperature (° C.)	Date
	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28						
Frequency (in number of worms)	...	5	12	53	71	116	157	245	292	379	348	202	72	26	14	7	...	1	...	...	...	...	...	2,000	15	14.4±0.05	21.7–29.4	25.55	July, 1944
	1	7	11	22	24	29	64	91	124	176	289	317	367	223	134	48	37	16	11	8	2	1	...	2,000	18	16.7±0.06	10.2–17.3	13.75	November, 1944
	...	2	3	5	7	14	27	42	71	135	224	296	324	368	226	121	73	29	18	7	4	3	1	2,000	19	17.9±0.06	5.1– 9.8	7.45	January, 1945

TABLE 2

VARIATION IN LEVEL OF LEAST SUSCEPTIBILITY TO POTASSIUM CYANIDE IN *Stylaria fossularis* AT DIFFERENT TEMPERATURES

	Level of Least Susceptibility (in Number of Trunk Segments Anterior to the Meeting Point)																	Total Number of Worms Examined	Empirical Peak	Mean	Temperature Range (° C.)	Average Temperature (° C.)	Date
	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19							
Frequency (in number of worms)	2	5	11	20	22	27	29	37	20	10	8	4	2	2	1	....		200	11	9.9±0.18	21.7–29.4	25.55	July, 1944
	1	4	5	7	14	18	23	28	34	22	15	11	9	5	3	1		200	12	11.4±0.20	10.1–16.6	13.35	November, 1944
	1	2	6	9	14	19	26	32	35	22	14	9	7	3	1	1		200	12	11.2±0.19	5.7–11.5	8.60	January, 1945

TABLE 3

VARIATION IN LEVEL OF LEAST SUSCEPTIBILITY TO MERCURIC CHLORIDE IN *Stylaria fossularis* AT DIFFERENT TEMPERATURES

	Level of Least Susceptibility (in Number of Trunk Segments Anterior to the Meeting Point)																Total Number of Worms Examined	Empirical Peak	Mean	Temperature Range (° C.)	Average Temperature (° C.)	Date
	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19					
Frequency (in number of worms)	4	5	9	11	16	20	22	26	31	22	14	7	4	4	3	2	1	200	11	9.9±0.20	19.4–26.3	22.85	September, 1944
	1	4	6	9	13	17	20	22	32	27	19	13	9	4	3	1	....	200	11	10.6±0.21	14.6–19.3	16.95	April, 1945
	1	2	3	7	11	15	19	25	30	33	21	13	8	6	3	2	1	200	12	10.9±0.20	6.9– 9.4	8.15	March, 1945

range from the sixth through the twenty-seventh trunk segment (second row in Table 1 and graph *B* in Fig. 1), and the empirical peak of the frequency curve has also shifted to the eighteenth segment.

The third series was done in January, 1945, and was finished within 14 days.

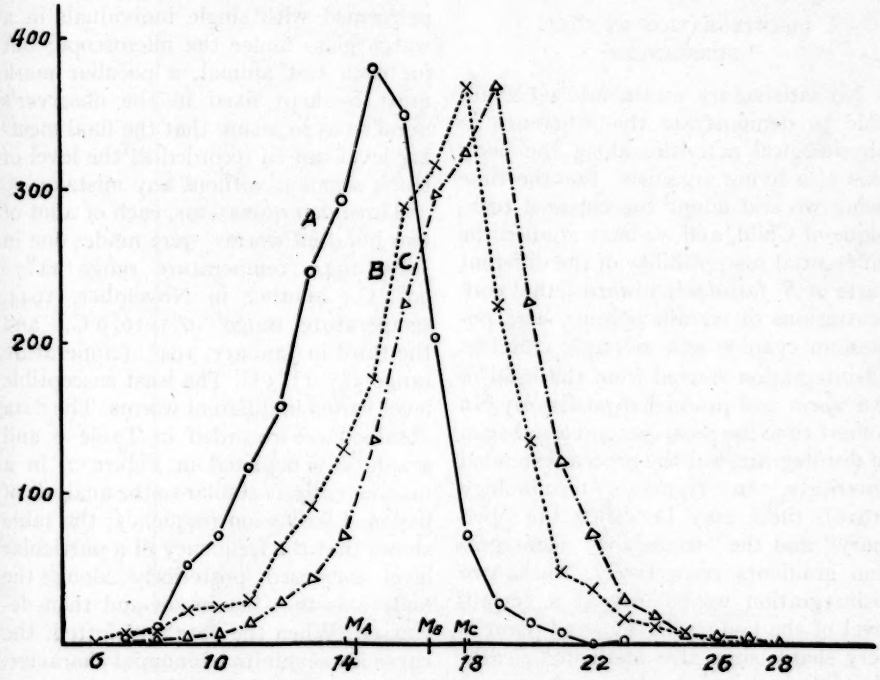

FIG. 1.—Fission frequency curves at different temperatures: *A*, 21°.7–29°.4 C.; *B*, 10°.2–17°.3 C.; and *C*, 5°.1–9°.8 C. Ordinates, numbers of animals; abscissae, numbers of trunk segments anterior to the fission zone.

The temperature ranged generally from 5°.1 to 9°.8 C. The results are shown in the last row of Table 1 and in graph *C* in Figure 1, which indicate a similar extent of fission-zone distribution and a further shift of the level of maximum fission frequency toward the posterior end.

From the data just given, it is very clear that temperature has a profound effect on the relative position of the fission zone. At higher temperatures (21°.7–29°.4 C.), the maximum frequency of fission appears at the more anterior trunk segment, and the range of distribution of the fission zone is comparatively narrow, including about seventeen trunk segments. The lowering of average temperatures by 12° C. (from 21°.7–29°.4 C. to 10°.2–17°.3 C.) causes a change of the empirical peak of the fission-frequency curve from the fifteenth to the eighteenth trunk segment and an extension of distribution of the zone from 17 to 22 trunk segments. A further lowering of 6° C. in average temperature (from 10°.2–17°.3 C. to 5°.1–9°.8 C.), however, produces but little effect, merely moving the peak of

frequency curve one more segment toward the posterior end. If these data were converted into their respective mean values, the orderly shifting of the peak of fission frequencies during the change of temperature is equally obvious, as is shown in the temperature columns of Table 1.

DISINTEGRATION BY TOXIC SUBSTANCES

No satisfactory means are yet available to demonstrate the difference in physiological activities along the body axis of a living organism. For the time being we still adopt the classical technique of Child, and we have studied the differential susceptibility of the different parts of *S. fossularis* toward lethal concentrations of certain poisons, e.g., potassium cyanide and mercuric chloride. Disintegration started from the head of the worm and proceeded posteriorly. In a short time the posterior end also began to disintegrate, but the process extended anteriorly. In Hyman's terminology (1916), these may be called the "primary" and the "secondary" disintegration gradients, respectively. These two disintegration waves met at a certain level of the body axis. The end-point is very sharp, since the death was clearly characterized by an abrupt change of body color from segment to segment, as was stated by Hyman.

The aim of the present experiment was to find out the meeting point of the two disintegration gradients, i.e., the level of least susceptibility, and to analyze the variations of such points within a given population. Moreover, since the temperature might well have some direct bearing upon the process of disintegration, the experiment was also carried out in three different periods, comparable to those during the analysis of the temperature effect of the fission-zone distribution.

1. *Cyanide treatment.*—The suitable concentration of potassium cyanide used in this experiment was M/50–M/80 solutions. A healthy, young worm without fission zone will be completely disintegrated in this solution within 3 hours. Disintegration experiments were performed with single individuals in a watch glass under the microscope; but for each test animal, a peculiar mark must be kept fixed in the observer's mind so as to insure that the final meeting level can be recorded as the level of trunk segment without any mistake.

Three determinations, each of a lot of two hundred worms, were made: one in July, 1944, temperature range 21°.7–29°.4 C.; another in November, 1944, temperature range 10°.1–16°.6 C.; and the third in January, 1945, temperature range 5°.7–11°.5 C. The least susceptible level varied in different worms. The data obtained are recorded in Table 2 and graphically depicted in Figure 2, in a manner entirely similar to the analysis of fission. Like fission frequency, the table shows that the frequency of a particular level increased posteriorly along the body axis to a maximum and then decreased. When the data are plotted, the curve also exhibits a binomial character. A similar, though less conspicuous, temperature effect on the level of the meeting point of the disintegration waves has been observed. Thus, for a lowering of 12° C., the corresponding shift of the maximum frequency was only one segment toward the posterior end of the worm. To explain this result, it should be noted that the worms used in these experiments were very young and, consequently, possessed relatively few trunk segments.

2. *Mercuric chloride treatment.*—Disintegration produced by mercuric chlo-

ride in *S. fossularis* was quite similar to that produced by potassium cyanide. The only difference was that, in the case of mercuric chloride, disintegration began after longer exposure but proceeded more rapidly than with cyanide. The favorable concentrations used were

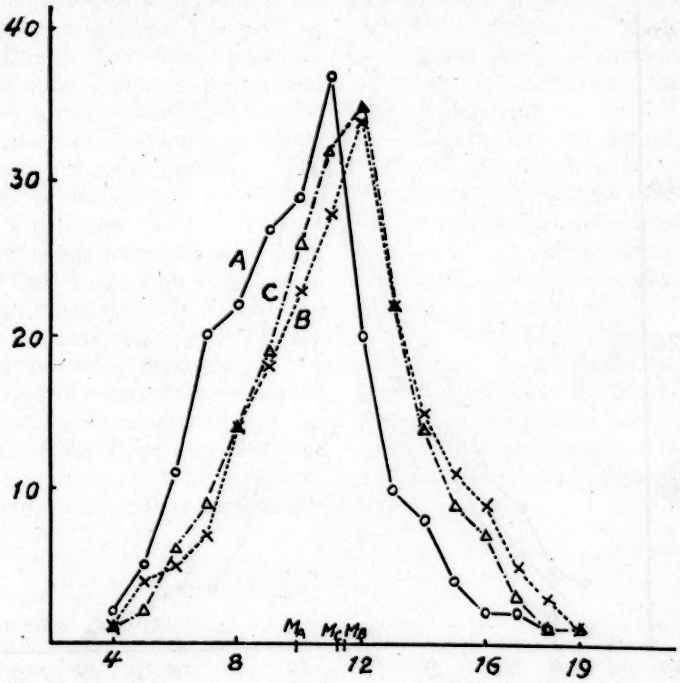

FIG. 2.—Frequency curves of the level of least susceptibility to potassium cyanide. *A*, 21°.7–29°.4 C.; *B*, 10°.1–16°.6 C.; *C*, 5°.7–11°.5 C. Ordinates, numbers of animals; abscissae, numbers of trunk segments anterior to level of least susceptibility.

M/150,000–M/250,000. The data are presented in Table 3 and Figure 3.

DISCUSSION

Literature discussing the factors which influence the fission process in annelids is extensive. Piquet (1906) and Schuster (1915) both reached the conclusion that, aside from the individual variation in the position of the fission zone, there is also a seasonal variation. Later, Stolte (1923) stated in a more definite way that in *Nais elinguis*, the value *N* (number of segments anterior to the fission zone) decreased in a natural population from 17 in January to 13.69 in March. It then rose rapidly in the sexual period (June–July) and declined again in the fall of the year. The latter author produced additional evidence in *N. variabilis* that the position of the fission zone was also dependent on the nutritive condition of the animal and that the extent of the effects of temperature and nutrition was largely conditioned by the age of the animal. Recently, Van Cleave (1937) arrived at a similar conclusion from his work in *Pristina longiseta*. The data presented here entirely confirm these views. There-

fore, it is fairly certain that the position of the fission zone in most annelids varies among different individuals and with different temperatures.

According to the work of Child's school, the higher metabolic rate of a part of an organism, the more susceptible a different trunk segment in different temperature ranges.

On account of the age difference between worms used for counts of fission frequency and for disintegration experiments, it seems very difficult to compare these results directly. But, from the gen-

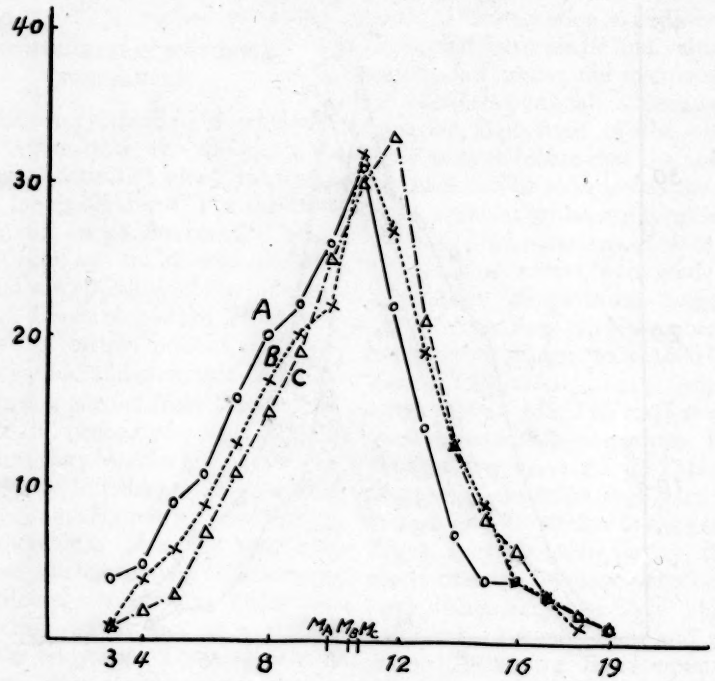

FIG. 3.—Frequency curves of the level of least susceptibility to mercuric chloride. *A*, 19°.4–26°.3 C.; *B*, 14°.6–19°.3 C.; *C*, 6°.9–9°.4 C. Ordinates, numbers of animals; abscissae, numbers of trunk segments anterior to level of least susceptibility.

to the toxic substances this part will be. Disintegration experiments in *S. fossularis* show that the posterior end of the worm is in a metabolic state nearly as high as its anterior end. The results are the same, whether we use potassium cyanide or mercuric chloride as the disintegration agent. Variation in temperature causes change of the level of least susceptibility, so that in a given population the peak of the frequency curve is at

eral nature of fission frequency and susceptibility curves, it may be suggested that the underlying causes which are responsible for the character of these curves appear to be similar. In other words, the physiological condition of the fission zone in a dividing worm may be somewhat similar to that of the level of least susceptibility in another worm. In counts of fission frequency, the observed worms are all mature, so that the

maximum frequency is always localized relatively far posteriorly. On the other hand, only those worms with fewer trunk segments and without fission zone were treated with toxic substances, so that the level of least susceptibility is more anterior. It appears probable that if we could use mature worms to perform the disintegration experiment, the peak of the susceptibility curve would approach that of the fission-frequency curve. Actually, this is not realizable, since the incipient fission zone of a mature worm often leads to a tertiary gradient of disintegration, as was noted by Hyman (1916) in *Dero limosa*.

Previous papers from this laboratory (Chu and Pai, 1945; Chu, 1946) have shown that natural fission in *S. fossularis* has some intimate relation to the anterior and posterior regeneration potencies. From the data here presented, we seem justified in suggesting that the phenomena of fission, regeneration, and disintegration may have the same physiological basis. In short, they are probably all expressions of the general physiological gradient pattern of *Stylaria* under somewhat different conditions.

SUMMARY

1. The distribution of the fission zone in *S. fossularis* is affected by temperature: at lower temperatures (1) the range of distribution becomes more extended and (2) the region of maximum fission frequency is shifted toward the posterior part of the worm.

2. Experiments on disintegration in *S. fossularis*, carried on with potassium cyanide and mercuric chloride, show that the position of the level of least susceptibility is also variable within a given population. Temperature affects the level in much the same way as it does the position of the fission zone.

3. Based upon the similarity of the general character of the fission frequency and the meeting-point curves, it is suggested that fission, regeneration, and disintegration must be in some way physiologically related.

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PHENOMENA THAT OCCUR IN THE CENTRIFUGED EGGS OF THE SILKWORM BOMBYX MORI L.

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PREVIOUS INVESTIGATIONS IN EXPERIMENTAL EMBRYOLOGY OF *Bombyx mori* L.

THE silkworm *Bombyx mori* L. supplied the material for what may be considered the first investigations on fertilization: the artificial parthenogenesis of silkworm eggs attempted by Tichomiroff (1885).

The eggs of the silkworm of univoltine races and of the last annual generation of bivoltine races have a short period of development after being laid (2–5 days, according to the temperature); thereafter the eggs experience a period of morphological inactivity, called "diapause," which lasts for some months in the cold season.

During this period the eggs present the following structure, from the outside toward the interior: (1) the chorion, which surrounds the entire egg and is composed of a substance chemically similar to keratine; (2) the egg membrane; and (3) the serosa, formed of large and flattened cells, with pigment granulations differently distributed according to the race (Tirelli, 1939). During the winter diapause in a monovoltine race the pigment is distributed around the membranes of each cell; in a bivoltine race the pigment is concentrated about the nucleus (Pl. I, *f*, *g*,). The interior of the egg is occupied by the yolk, in which we observe some nuclei of irregular shape, isolated or in groups of two to four, generally surrounded by a circular zone of vacuoles. Nuclei and vacuoles form the yolk cells (Tirelli, 1930).

The embryo appears as a stripe composed of two cellular strata: a stratum of cylindrical ectodermal cells and a stratum formed by fourteen groups of mesodermal cells turned toward the interior of the egg (Pl. I, *h*) (Tirelli, 1930).

The cephalic end of the embryonal stripe is always turned toward the micropyle, and this fact has been interpreted as an index of the predetermination of the eggs. The ectodermal stratum of the embryonic stripe is protected by a membrane, the amnion, originated especially by emigrating yolk cells (Grandori, 1915); this stratum forms the amniotic cavity, containing the amniotic liquid, between the ectodermal side of the stripe and the inside of the amnion.

Some eggs, during the diapause, show a sinuous embryonic stripe, which is deep in the yolk (Grandori, 1928–29; Tonon, 1925). Let us keep in mind the structures briefly described for comparison with those of the centrifuged eggs.

In the spring, after the diapause, the eggs continue their development. It has long been known in Italy that by brushing the eggs of the silkworm, immediately after laying, with branches of *Erica* a considerable percentage of eggs develops without diapause (Barca, 1872). Similar results are obtainable with so-called "electric rain" and also by a short immersion of the eggs in an acid, preferably concentrated hydrochloric acid, or in hot water (80° C.) The treated eggs show a continuous development, as do the eggs of nonhibernating generations of the

bivoltine and polyvoltine races (Verson, 1878; Quaiat, 1913).

The unfertilized eggs of the silkworm always show a natural tendency to parthenogenesis, greater in the bi- and multivoltine than in the univoltine races. This phenomenon was the cause of a long discussion as to whether unfertilized eggs do or do not produce living larvae. The problem was positively resolved by Bugini (1929–30); and the morphological and physiological phenomena which occur in the parthenogenetic eggs have been studied by Iucci (1926), Grandori (1924), and Niceta (1928–29), among the more recent investigators.

Tichomiroff (1885) successfully applied the methods used to suppress the diapause in activating unfertilized eggs. By treating the unfertilized eggs with hydrochloric acid or by brushing the eggs he obtained a larger percentage of partially developed eggs than in the control.

After this interesting beginning, the eggs of the silkworm were not used by others as material for investigations on experimental embryology, largely because of technical difficulties resulting from the structure of the eggs. Studies of the factors that suppress the diapause alone were continued, particularly for their practical applications. Centrifuging was applied by Clement (1920*a*, *b*) to mature silkworms, producing some abnormalities in the metamorphosis, and by Kawaguchi (1936, 1938*a*, *b*) to the eggs. Kawaguchi (1936) centrifuged (10 minutes at 3,500 turns, with 16-cm. pole) silkworm eggs from 1–3 hours after laying—that is, during the maturation and the zygosis of the pronuclei, according to his previous observations (Kawaguchi, 1928). The centrifuged eggs had a known genetic patrimony and should have produced the following silkworms: (1) Moricaud striped, white haemolymph; and (2) clear striped, yellow haemolymph, in determined proportion. The centrifuged eggs also produced such unexpected combinations as (1) Moricaud striped, yellow haemolymph; (2) very clear striped, yellow; etc. Kawaguchi (1936, p. 115) thinks that the unexpected combinations resulted from fusion of the egg nucleus with two spermatozoa, forming triploid individuals. The triploid male moths are not fertile; the females are fertile in small numbers.

The centrifuged eggs also produced tetraploid individuals. Tetraploid females, fertilized by diploid males, are fertile (79.6 per cent); the matings of tetraploid males with diploid females are fertile (18.8 per cent); and those of tetraploid males with tetraploid females have a similar fertility (16.6 per cent) (Kawaguchi, 1936, p. 131).

The triploid individuals possess ovaries sometimes smaller than the diploid and lay eggs, larger or smaller than the normal individuals, in an irregular manner. The tetraploid females always lay larger eggs than do the diploid.

Kawaguchi does not analyze the embryological phenomena in the centrifuged eggs because centrifuging for only 10 minutes, during fertilization or maturation, as he did, does not change deeply the embryonic structures not yet formed or the distribution of the substances of the yolk.

TECHNIQUES OF THE PRESENT EXPERIMENTS

The eggs, flattened and adhering to paper where laid, were centrifuged in different directions, with the position of the eggs in the tube of the centrifuge being varied.

Some experiments had been carried out without controls; in other experiments each laying was divided into sev-

eral portions—one was the control, the others were centrifuged in various directions and for different periods. Some eggs were fixed after the centrifugation, while others were examined during various stages of their development.

Since centrifuging with 17-cm. pole, 5,000 turns per minute, killed a great quantity of eggs, we used 2,500 turns per minute. The eggs, taken 2–4 hours or more after laying, were centrifuged continually for a few hours up to 2 or 3 days, in order to continue the modifying action of the centrifugal force during the same differentiation of the embryonic structures.

All the experiments were carried out with a yellow Italian race of silkworms.

The centrifuged eggs were immersed in 80° (sp. gr. Baumé scale) alcohol and examined, while alive, in the cavity of a Koch glass covered with a cover glass. Immersion in alcohol does not injure the eggs if the treatment is short (Quaiat, 1902). Other eggs were examined *in toto*, fixed, and mounted in Canada balsam.

Examination of the living eggs enables one to observe the modifications of the serosa and the stratification of the yolk. For the histological observations, the eggs were fixed with Carnoy; the shells were removed with Rusma (2 parts of CaO, 1 part of As_2S_3), according to Niceta (1929) and Tirelli (1935); and paraffin-sectioned, and dyed with Carazzi haematoxylin and eosin.

RESULTS AND OBSERVATIONS

A. MODIFICATIONS OF THE SEROSA AND DISPLACEMENT OF YOLK SUBSTANCES

Silkworm eggs centrifuged during the diapause, 2 months after laying, present as the most characteristic modification the displacement of the pigment of the serosa toward the centrifugal edge of each cell. This phenomenon also permits an indirect valuation of the viscosity of the plasma, by the time necessary to displace the pigment to the centrifugal edge of the cells. Some incidental observations indicate that the viscosity of the cells of the serosa is variable in the different living eggs and is greater in diapause than during the first stages of development of the egg.

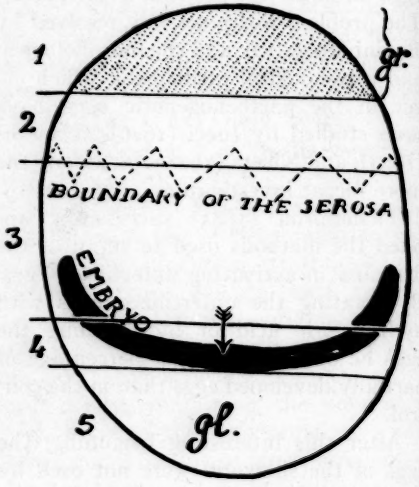

FIG. 1.—A schema indicating the stratification of the centrifuged eggs. Compare this schema with Plate II, *c*. Key: *1, 2, 3, 4, 5* = the five zones of stratification; *gr.* = fat zone; *gl.* = glycogen zone. The dotted line indicates the boundary of the serosa during the centrifuging of the egg. The arrow indicates the direction of the centrifugal force.

The most important results are observed in eggs centrifuged during the period of the multiplication of the blastomeres and the differentiation of the embryonic tissues up to the time of the formation of the embryonic stripe, beginning the centrifugation 2–8 hours after oviposition and continuing for 1–3 days. By centrifuging recently laid fertilized eggs, five well-defined strata may be observed—as early as 10–12 hours in some cases and after 15–24 hours in all centrifuged eggs (Pl. II, *c*; Fig. 1).

From the centripetal edge we observe,

in the living eggs examined immersed in alcohol: (*1*) an obscure cap; (*2*) a clear zone, finely granular, passing gradually into (*3*) an obscure zone; (*4*) a clear zone, finely granular; and (*5*) at the centrifugal edge a hyaline cap.

The boundary between the centrifugal cap and zone *4* is well defined (Pl. II, *b*, *c*; Fig. 1), as is the boundary between the centripetal cap and zone *2*. The boundaries between the other zones are not so clear. In the fixed eggs the boundary between zones *3* and *4* is also distinct (Pl. II, *b*). The centrifugal cap can be observed only in the eggs centrifuged during a long period; otherwise, this cap (zone *5*) and zone *4* are mixed in one zone only: then in the eggs there are visible four layers instead of five.

Between zones *2* and *3*, in the eggs examined *in toto*, a red or red-brown spot can be observed (Pl. II, *a*, *b*, *c*), formed by a store of serosa pigment. This spot affects only the surface of the eggs; it appears in sections as a deposit of pigment, with granulations closely in contact; cellular limits are not visible.

The hyaline centrifugal cap of eggs centrifuged 10 hours or more after laying and fixed immediately after centrifugation present a clear reaction of glycogen (brown-red coloration with Lugol's liquid) (Tirelli, 1931). The centripetal cap (zone *1*) can be stained with Sudan III (fat). The intermediate zones, *2*, *3*, and *4*, present the protein reactions—xantoproteic and Millon's—while caps *1* and *5* do not present similar reactions. Zones *2*, *3*, and *4* constitute a protein-water region.

Coloration with Sudan III is better observed in the eggs after removing the shells without previous fixing. Kawaguchi (1936, Fig. 2) presents a semischematic figure of an egg centrifuged 20 hours—with a fat zone, a yolk zone, and a plasma zone, in the centrifugal edge. This last zone corresponds to the glycogen cap rather than to a plasma zone. Bodine and Boell (1936) present a figure of a centrifuged grasshopper egg stratified in four zones that correspond to our results.

In eggs centrifuged continually 10 or more hours we observe the formation of

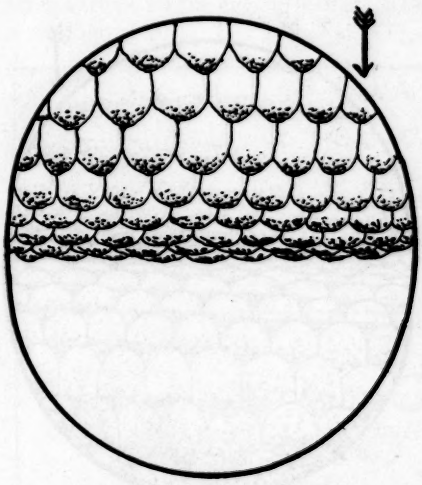

FIG. 2.—Schema of the disposition of the serosa cells in an egg centrifuged after laying, in which the serosa has been formed during the centrifuging, and fixed immediately after centrifuging. Arrow indicates direction of centrifuging.

an abnormal serosa. The cells of the serosa are formed only in the centripetal edge and appear long; in each cell the pigment is displaced in the centrifugal edge. If the centrifugation is continued, the serosa cannot wrap around the egg but stays on the centripetal edge, as a cap (Pl. II, *a*), which presents a ring of cells compressed on each other (Pl. II, *e*; Fig. 2).

After the centrifuging is completed, the serosa takes its normal structure: the long and flat cells of the centripetal edge become shorter; the compressed and thick cells of the marginal ring cover all the surface of the egg (Figs. 3 and 4);

and in each cell the pigment is distributed as in the normal eggs. Sometimes a little store of pigment remains in the centrifugal edge of each cell for a short time. The possibilities of pigment behavior have been demonstrated by Tirelli (1934) in normal eggs. The emigrating cells of the serosa present club

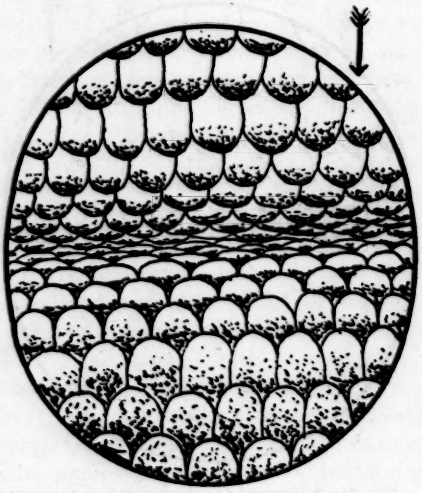

FIG. 3.—Schema of an egg centrifuged after laying, until the formation of the serosa, and fixed some hours after centrifuging. The serosa cells again cover all the egg surface. Arrow indicates direction of centrifuging. Compare this schema with Plate II, *d*.

forms, as in the esticotropism of Ruffini (1925) (Fig. 4).

If the centrifugation is accomplished in eggs laid 2 or 3 days previously, when the serosa is already formed and begins to become red, the serosa is sometimes broken; and there occur the same phenomena as in eggs centrifuged immediately after laying. The larger surface of the cells at the centripetal edge is not produced by greater volume of cells but because the cells become larger and thinner. In correspondence with the terminal ring, the cells are thick.

B. YOLK CELLS

In sections of eggs which were centrifuged 10 or more hours after laying and fixed after the centrifuging, we observe a spongy zone, corresponding to cap *1*, that presents numerous vacuoles separated by filaments of plasma, with some nuclei similar to the nuclei of the normal yolk cells. Sometimes a zone of smaller vacuoles and a zone of larger vacuoles

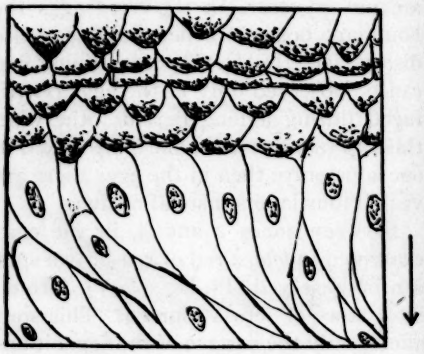

FIG. 4.—Serosa cells (compressed by the centrifuging) that emigrate and present elongated forms.

can be observed at the extreme centripetal cap. Some nuclei also appear in the intermediate zones of the egg.

We can understand the origin of the vacuolar cap by observing the structure of the normal eggs. The yolk is partially formed by yolk cells, composed of one or more nuclei surrounded by a ring of vacuoles, which are surrounded by a homogeneous yolk (Tirelli, 1929, 1930). In the centrifuged eggs (cf. Pl. I, *h*, with I, *d*) the vacuoles of the yolk cells are displaced to the centripetal edge, and the nuclei of the yolk cells remain isolated in the homogeneous yolk of zones *2*, *3*, and *4* or in the plasma filaments that limit the vacuoles in the centripetal cap.

During the centrifuging or the natural stratification of the sap extracted from the eggs we observe (Tirelli, 1929,

1936) the separation of an upper gray layer, with fat and lipoid substances making up about one-third of the total volume of sap in the tube. The vacuoles displaced to the centripetal edge of the egg occupy about one-third of the volume of the egg; the vacuoles probably contain fatty substances or lipoids.

Incinerated sections of the centrifuged eggs demonstrate a greater quantity of ash in cap *5*, while the vacuoles of zone *1* appear empty (Pl. I, *e*).

C. EMBRYO

In eggs centrifuged 2 or more days after laying and fixed after centrifugation the embryo appears in zone *4*. Sometimes the embryo appears in the middle of the protein region, that is, in zone *2* or *3* (Pl. I, *d*). Many eggs present the embryo rolled at the edge like a ring, with the ectoderm exterior, the mesoderm interior (Pl. I, *c*); and the symbionts of Grandori (1919–20) appearing in the interior of the rolled embryos are absent or very rare in the yolk that surrounds the embryo. In some eggs, particularly in the protein zones *2*, *3*, and *4*, there can be observed numerous round nuclei, very different from the nuclei of the yolk cells, which are smaller and of irregular form, and from the round but smaller nuclei of the embryo. Perhaps these nuclei (Pl. I, *a*, *b*; Fig. 5) are nuclei of the serosa displaced into the interior of the egg and are spherical instead of round and flattened in form. This fact confirms the hypothesis of a predetermination of the blastomeres; thus, the nuclei of the serosa appear different from those that form the yolk cells or the embryo, and this difference also exists when the centrifugation displaces the nuclei into a zone other than that to which they were destined.

The centrifugal edge of the eggs appears regularly convex in sections; the centripetal vacuolar zone is often deformed, with the external surface of the egg wrinkled, on account of the stretching of the cells for the pigment (Pl. I, *d*).

The embryonic structure having the highest possibility of regulation is the serosa, the cells of which, displaced at the centripetal cap by the centrifugation, again inclose all the egg surface after the centrifuging.

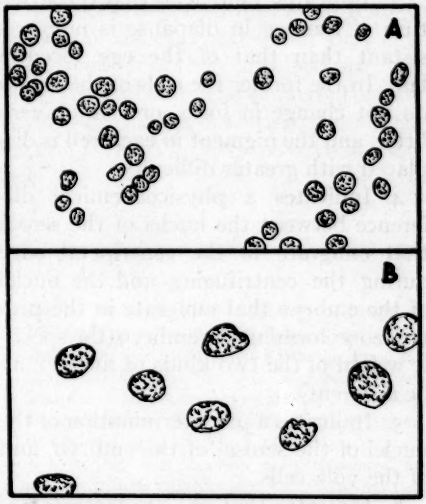

FIG. 5.—Schema of the size of the nuclei in Plate I, *a*, *b*, which demonstrates clearly their different size. *A* = embryonic nuclei; *B* = serosa nuclei displaced in the yolk.

The stratification of the substances of the yolk continues to remain in the eggs during the diapause. Some 50 per cent or more of the centrifuged eggs hatch, and the silkworms do not generally present abnormalities during their development. In some instances we have observed a greater mortality at the most advanced age, and also some larvae which were smaller than normal.

DISCUSSION AND SUMMARY

From the point of view of structure and of chemical phenomena in the eggs, the present work—

1. Confirms the fatty or lipoidic nature of the content of the vacuoles that surround the nuclei of the yolk cells and the considerable quantity of glycogen in the yolk.

2. Indicates some properties of the serosa, the cells of which can change in form and can emigrate, with the displaced pigment taking the primitive position in the cell.

3. Indicates, moreover, that the structure of the egg in diapause is more resistant than that of the egg recently laid. In the former the cells of the serosa do not change in form, or change very little; and the pigment in each cell is displaced with greater difficulty.

4. Indicates a physicochemical difference between the nuclei of the serosa that emigrate to the centripetal edge during the centrifuging and the nuclei of the embryo that emigrate in the protein zone, forming the embryo (the specific weight of the two kinds of nuclei may be different).

5. Indicates a predetermination of the nuclei of the serosa, of the embryo, and of the yolk cells.

6. Indicates that the displacement of the embryo in an abnormal position does not determine an inhibition of its development. A regulation of the position of the embryo occurs at the end of the diapause.

7. Indicates that the complete stratification of the yolk, the separation of its constituents, and the destruction of its normal morphology—i.e., the separation of the fatty vacuoles from the yolk cells—do not determine an inhibition of development.

The principal phenomena which occur in the centrifuged eggs of the silkworm *Bombyx mori* L. are the following:

1. *Serosa.*—In eggs taken 2–4 hours after laying and centrifuged for 2–3 days, that is, during the embryonic development, the serosa appears in the centripetal edge of the eggs (Pl. II, *a;* Fig. 2); its cells are thin and elongated in the centripetal cap, larger and thicker at the border (Pl. II, *e*). In each cell the pigment is displaced to the centrifugal edge (Pl. II, *d*, *e*). On completing the centrifugation the cells of the centripetal cap become shorter, while the cells of the terminal ring emigrate and cover the entire surface of the egg (Fig. 4); the pigment now takes its characteristic distribution.

In eggs centrifuged 2–5 days after laying, when the blastoderm is already formed or when the serosa is forming, the centrifugation sometimes breaks the serosa, and the phenomena just described can be observed. If the centrifuging does not break the serosa, an elongation of the cells in the centripetal cap is especially noteworthy.

In eggs centrifuged during the diapause the cells of the serosa do not present changes of form and the pigment is always displaced to the centrifugal edge of each cell, but with more difficulty and not so completely as in the eggs centrifuged shortly after laying. The cytoplasmic viscosity of the serosa cells is greater in the eggs during the winter diapause than it is in eggs just laid.

2. *Yolk.*—In unfertilized and fertilized eggs, centrifuging produces an evident stratification of the yolk. In the fertilized eggs five zones are formed—the one at the centrifugal edge consisting of glycogen, that of the centripetal edge, of fatty substances. The intermediate zones present the protein reactions and constitute a protein region (Pl. II, *a*, *b*, *c;* Fig. 1).

In the sections we observe a vacuolar zone in the centripetal edge (Pl. I, *d*), the vacuoles of which may be considered as the vacuoles that surround the nuclei of the yolk cells in the normal eggs. These

vacuoles are separated by filaments in which we observe some nuclei, interpreted as nuclei of the yolk cells.

In some eggs, centrifuged for about 24 hours, 1–2 days after being laid, we observe some large round nuclei in the protein zone, which can be considered as nuclei of the serosa displaced into the interior of the eggs by the centrifugation (Pl. I, *a*, *b*; Fig. 5).

On completing the centrifugation the stratification of the yolk remains a long time, until the ulterior development of the embryonic stripe.

3. *Embryo.*—The embryos of the eggs centrifuged some hours after laying, or 2–3 days later, appear displaced toward the centrifugal edge in the protein region (Pl. I, *d*). The embryos are sometimes more or less rolled up at the edges, with the ectoderm on the exterior side (Pl. I, *c*).

After centrifugation, some embryos remain displaced for a long time during the diapause; the regulation of their position probably occurs when their development begins in the spring; more than 50 per cent of the eggs hatch, and some eggs do not complete their development.

All these phenomena indicate that the ideas concerning predetermination of the eggs of *B. mori* L. may have to be modified.

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PLATE I

FIG. *a*.—Nuclei of the embryo of a silkworm.

FIG. *b*.—Bigger nuclei (serosa nuclei displaced) in the yolk at same magnification.

FIG. *c*.—An embryonic stripe rolled in a centrifuged egg. The symbionts of Grandori are in the interior of the embryo.

FIG. *d*.—Section of a centrifuged egg. *St* = embryonic stripe; the vacuolar zone is the fatty zone.

FIG. *e*.—The ash of a section of a centrifuged egg. The ashes are more abundant in the glycogen zone—that is, the part opposite to the vacuolar zone or fatty zone.

FIG. *f*.—Normal serosa of a wintering egg in diapause of a monovoltine silkworm race. The pigment is distributed, in each cell, around the membranes.

FIG. *g*.—Normal aspect of a wintering egg of a bivoltine silkworm race. The pigment is distributed around the nuclei.

FIG. *h*.—Section of an egg of silkworm in the embryonic-stripe stage. *S* = embryonic stripe; *C* = yolk cells with nuclei and vacuoles.

PLATE II

FIG. *a*.—A silkworm egg centrifuged for 1 day after laying, until the formation of the serosa. *gr* = fat zone; *gl* = glycogen zone; *P* = red pigment mass. The serosa cells are visible in the fat zone. The internal arrow indicates the marginal ring of compressed serosa cells; the external arrow, the direction of the centrifuging. The egg was fixed immediately after the centrifugation and mounted in Canada balsam.

FIG. *b*.—A silkworm egg fertilized and centrifuged during first day after laying. *1* = fat zone; *2*, *3*, *4* = protein zones; *5* = glycogen zone; *pig.* = red pigment mass. The arrow indicates the direction of the centrifugal force. The egg has been fixed immediately after centrifuging and mounted in Canada balsam.

FIG. *c*.—An egg of silkworm centrifuged during first day after laying. *1*, *2*, *3*, *4*, *5* = the five zones of stratification (*1* = fat zone; *2*, *3*, *4* = protein zones; *5* = glycogen zone); *pig.* = red pigment masses; *gl* = glycogen; *p* = limits of the protein zones; *C* = yolk cells. The arrow indicates the direction of the centrifugal force. The egg is still living (difference from egg in *b* observed in Canada balsam).

FIG. *d*.—A centrifuged egg with the equatorial ring of the compressed serosa cells. The superior half of the egg is clearer because it is the fat-vacuolar zone. In each serosa cell the pigment is displaced to the centrifugal edge. The arrow indicates the direction of the centrifugal force. The egg has been fixed and mounted in Canada balsam after the centrifuging (compare with text Fig. 3).

FIG. *e*.—Portion of serosa of a centrifuged egg, fixed immediately after centrifuging. In each cell the pigment is clearly displaced to the centrifugal edge. The cells are flattened and large in the upper portion and compressed in a ring of short cells in the inferior portion. The black arrow indicates the direction of the centrifuging; the small white arrows indicate mass of pigment put forward from some serosa cells.

PLATE I

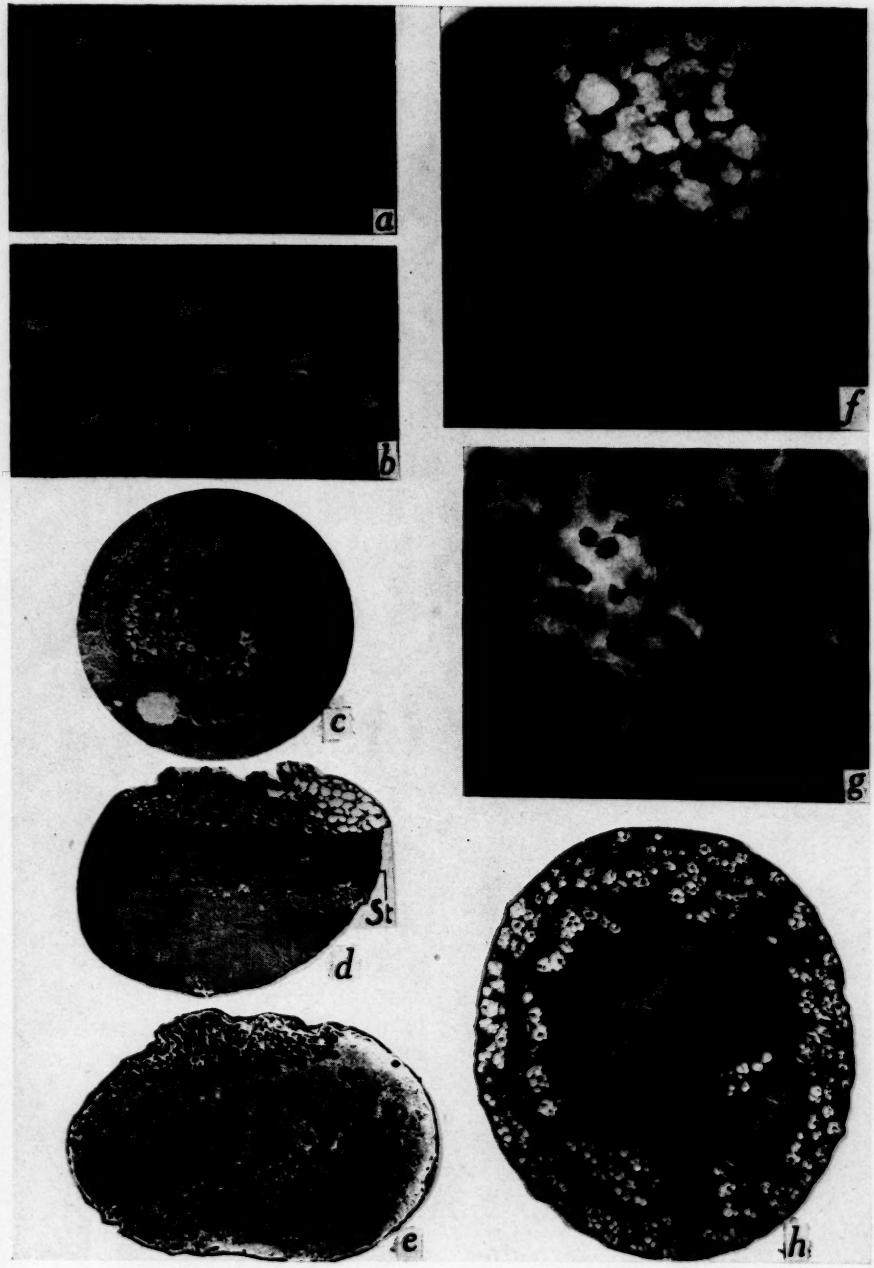
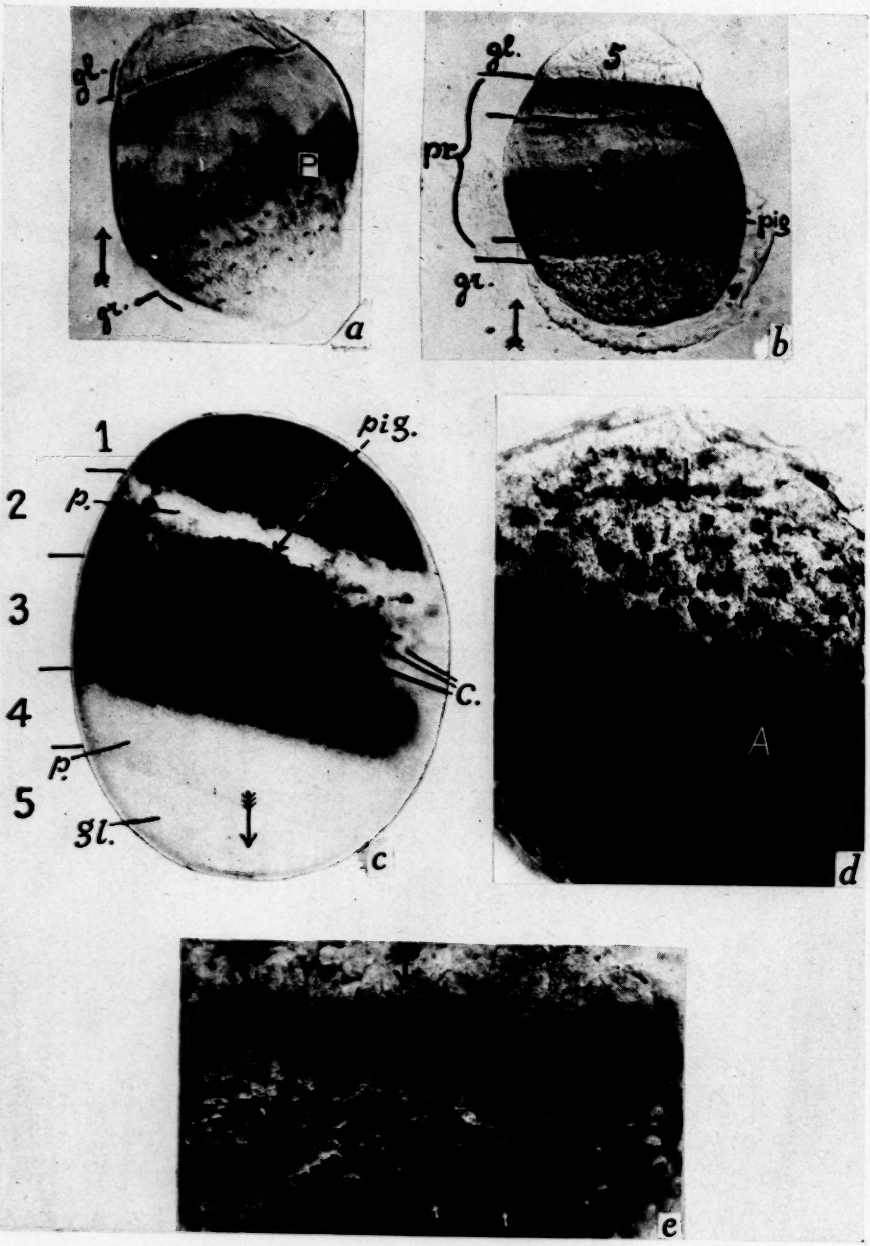


PLATE II



THE EFFECT OF PEANUT OIL ON THE DESICCATION OF DIAPAUSE AND POSTDIAPAUSE PUPAE OF THE MOTH, CALLOSAMIA PROMETHEA DRURY

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THE cocoons of the Promethea moth are suspended from the branches of trees, where the pupae are exposed to the drying effects of the atmosphere throughout the winter and spring months. Preliminary experiments have indicated that these pupae are very resistant to desiccation and are able to survive exposures of several months over anhydrous $CaCl_2$ at 25° C. Thus, in respect to habitat and resistance to water loss, they are similar to the pupae of the Cynthia moth, *Samia walkeri* (Ludwig, 1943). A series of experiments is now in progress to determine the factors responsible for the ability of these insects to resist dehydration in dry air. Information regarding the probable nature of these factors was obtained from a paper by Ramsay (1935), who found that the evaporation of water from the cockroach, *Periplaneta americana*, was modified by the presence of a film of some fatty substance, which undergoes a change of state at about 30° C. He observed that, above this temperature, the rate of evaporation increased enormously with increasing temperature. These results were obtained with living or dead animals, whose spiracles were blocked with wax. Hence the water was lost through the body surface, and the increase in rate above 30° C. was believed by Ramsay to be associated with the melting-point of a fat which forms a film covering the surface of the cuticle. Ludwig (1946) observed that the rate of dehydration of Japanese beetle pupae was greatly increased by treating them with peanut oil or with peanut-oil solutions of D.D.T. He suggested that the oil might affect the water permeability of the cuticle by dissolving a waxy covering from the surface of the pupa. The present experiments deal with the changes induced in the water permeability of the cuticle of Promethea pupae by treatment with peanut oil.

MATERIAL AND METHODS

Promethea cocoons were collected in New York City. The pupae were removed from their cocoons and placed in glass vials, weighed on a chainomatic balance which was sensitive to 0.1 mg., and transferred to desiccators over anhydrous $CaCl_2$. To test the effect of temperature on the water permeability of the cuticle, series of pupae collected in March, 1945, were placed at 25°, 30°, and 35° C. Weight readings were made daily until the moths emerged. These pupae were found to be in the postdiapause state, many moths emerging in 2–3 weeks at 25° and 30° C. In 1946, diapause pupae were collected early in February and placed at 20°, 25°, 30°, and 35° C., to compare results with those obtained previously with developing pupae.

To determine whether peanut oil affects the rate of dehydration, diapause pupae were rolled on the surface of filter paper moistened with peanut oil, so that the cuticle was thoroughly coated with

the oil. At the end of 10 minutes, the oil was cleaned from the surface of the pupae. They were then weighed and placed in desiccators over anhydrous $CaCl_2$. Groups of diapause pupae (collected early in February, 1946) were given only one treatment with peanut oil at the beginning of the experiment and were then placed at 20°, 25°, 30°, and 35° C. Other groups of diapause and post-diapause pupae (collected in March, 1946) were placed in desiccators over anhydrous $CaCl_2$ at the temperatures indicated above and were treated daily with peanut oil. The pupae of all series were weighed daily throughout the course of the experiment.

OBSERVATIONS

In Figure 1 are shown the rates at which normal and treated diapause pupae lost weight over anhydrous $CaCl_2$. Graph *A* shows the rates of weight loss of normal pupae. The saturation deficiency of the air in the desiccators is greater, the higher the temperature, being approximately 17.0 mm. of mercury at 20°, 23.4 mm. at 25°, 31.4 mm. at 30°, and 41.0 mm. at 35° C. Since the evaporating power of the air is proportional to the saturation deficiency, the pupae should lose weight at a much greater rate at the higher temperatures. However, the figure shows that all normal pupae lost weight at the same rate at each of the four temperatures, the curves being so close together as to be practically indistinguishable. Furthermore, the rate of weight loss was extremely slow, the weights at the end of 18 days in dry air being approximately 94 per cent of their original value, regardless of saturation deficiency.

Graph *B* in Figure 1 shows the rates of dehydration of pupae given a single treatment with peanut oil. In this series there was an immediate separation of the rates into four groups, depending on saturation deficiency. Those exposed to the higher saturation deficiencies (higher temperatures) lost weight initially at a much greater rate than those exposed to the lower saturation deficiencies. However, the differences in rate persisted for only 3 or 4 days, following which the weight loss occurred at approximately the same rate as in normal pupae under

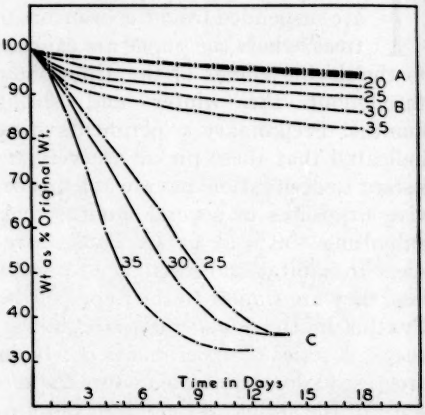

FIG. 1.—Rate of desiccation of diapause pupae over anhydrous $CaCl_2$ at different temperatures. Graph *A*, normal pupae; graph *B*, pupae given one treatment with peanut oil; graph *C*, pupae given daily treatments with peanut oil. Numbers indicate temperatures in degrees Centigrade.

the same conditions. This observation may indicate that the hypodermis of the pupa is able to restore the water impermeability of the cuticle after its impairment by treatment with peanut oil.

Graph *C* in Figure 1 represents the results of daily treatment with peanut oil on the rate of dehydration. At each saturation deficiency, this rate was accelerated with each of the first two or three exposures to oil. Weight loss then continued at a very rapid rate, until the pupae had lost more than 50 per cent of their original weight. This rapid loss of weight was followed by a gradual retardation of the rate until the weight re-

mained constant. The final weights cannot be used as an indication of the dry weight of the pupa, since a considerable amount of oil was undoubtedly left on the surface, or it may even have been absorbed by the cuticle as a result of the successive exposures. Peanut oil appears to be nontoxic, because nearly all the pupae exposed a single time produced normal adults; and, with successive treatments, pupae were relatively active for as long as 4 or 5 days, and apparently died of desiccation.

The graphs in Figure 2 show the rates at which normal and treated postdiapause pupae lost weight at the different temperatures. The upper curves are for normal pupae and, as in the case of diapause pupae, there is no difference in the rate of weight loss at the different saturation deficiencies. However, under similar conditions the postdiapause pupae lost weight at a much faster rate than did the diapause pupae. At the end of 12 days over anhydrous $CaCl_2$, the former group weighed approximately 85 per cent of their original weight; whereas, after the same period of time, this value for the latter group was more than 95 per cent.

The lower graph in Figure 2 represents the rates of weight loss of postdiapause pupae exposed daily to peanut oil. The results are similar to those just described for similarly treated diapause pupae, except that dehydration occurred at a more rapid rate.

DISCUSSION

It is evident from these observations that the "law of saturation deficiency" proposed by Mellanby (1935) does not apply to the loss of water from normal diapause or postdiapause Promethea pupae kept in dry air at different temperatures. According to this "law," insects of the same species in identical morphological and physiological states should lose water at a rate proportional to the saturation deficiency of the air, provided that the saturation deficiency is not above a maximum figure which is peculiar to the species. However, as shown in Figures 1 and 2, normal pupae lost weight at the same rate, regardless of saturation deficiency. This observation may indicate that the cuticle of the normal pupa is impermeable to water and

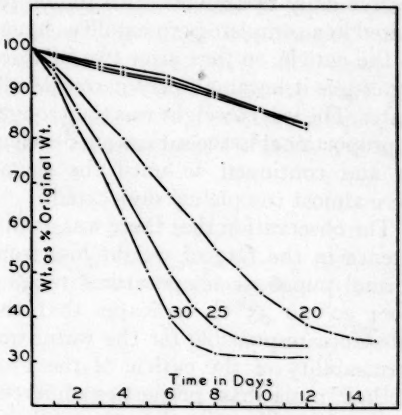

FIG. 2.—Rate of desiccation of postdiapause pupae over anhydrous $CaCl_2$ at different temperatures. *Upper graph*, normal pupae; *lower graph*, pupae given daily treatments with peanut oil. Numbers indicate temperatures in degrees Centigrade.

prevents the free evaporation of water from the surface of the pupa. It is also interesting to note that postdiapause or developing pupae lost weight at a faster rate than did diapause pupae under the same temperature and humidity conditions. This observation is in agreement with the results described by Ludwig (1943) for the pupae of the Cynthia moth. Presumably, the postdiapause pupae produce more water by their increased metabolism than is produced by diapause individuals, and this water is then lost by transpiration through the tracheal system.

The observation that a single exposure to peanut oil caused an increase in the

rate of evaporation of water from the treated pupae suggests that a substance soluble in peanut oil is present in the cuticle or covering its surface and that this substance is at least partially removed or changed by the oil. Its partial removal or modification resulted in a temporary increase in the rate of evaporation from the treated pupae, following which the original rate was restored. Successive daily treatments with the oil resulted in a complete permeability change in the cuticle, so that after two or three exposures it became freely permeable to water. The loss of weight was then roughly proportional to the saturation deficiency and continued so until the pupae were almost completely desiccated.

The observation that there was no difference in the rate of weight loss from normal pupae at temperatures ranging from 20° to 35° C. indicates that the substance responsible for the water impermeability of the cuticle of the Promethea pupa has properties different from that present on the cuticle of the cockroach, *P. americana*, and described by Ramsay (1935). He found a marked increase in the rate of evaporation from the cockroach at temperatures above 30° C., whereas, in the present experiments, no increase in rate of weight loss was observed at temperatures up to 35° C. No higher temperatures were employed, because 35° was found to be above the temperature which permitted the emergence of adults.

Mellanby (1934) performed a series of experiments to determine the site of water loss from an insect when kept in dry air. He used larvae of the meal worm, *Tenebrio molitor*, and of the clothes moth, *Tineola bisselliella*, and larvae and adults of the flea, *Xenopsylla cheopis*, comparing the rate of water loss from normal insects and from others kept in gas mixtures (such as air plus 5 per cent CO_2) which caused the insects to keep their spiracles permanently open. Insects treated with these gas mixtures lost water from two to seven times as fast as normal ones. He concluded that practically all the water lost by the insect evaporates through the tracheal system and that the cuticle is practically impermeable to water. However, the work of Ramsay (1935) shows that under certain conditions, such as exposure to high temperatures, the cuticle may become freely permeable to water. In the present experiments it appears likely that the loss of water from normal pupae occurred through the tracheal system but that after treatment with peanut oil the cuticle furnished the principal site for evaporation. The oil might bring about this change in the water permeability of the cuticle by removing or changing a fatty or waxy covering from its surface. Ludwig (1946) found that peanut oil had no effect on the rate of evaporation from Japanese beetle larvae and adults but that it increased the rate of water loss from the pupae. If the above explanation is correct, then it must be assumed that the cuticle of the former stages does not contain a substance affected by peanut oil; whereas the cuticle of the pupa does contain a peanut-oil-soluble material which retards or prevents evaporation. An attempt to isolate and analyze this substance from both Promethea and Japanese beetle pupae is planned for future experiments.

SUMMARY

1. Normal diapause Promethea pupae lost weight over anhydrous $CaCl_2$ at the same rate between the temperatures of 20° and 35° C., regardless of saturation deficiency. Postdiapause pupae, under the same conditions, lost weight at a rate which is also independent of saturation deficiency but which is more rapid

than that for diapause individuals. It is suggested that developing pupae produce more water as a result of their increased metabolism than is produced by diapause insects and that this water is lost by evaporation.

2. The rate of weight loss of both normal diapause and postdiapause pupae is very slow. In the former group the weight at the end of 12 days' exposure to dry air was more than 95 per cent of its original value, and in the latter group this value was approximately 85 per cent.

3. A single exposure of pupae to peanut oil results in a temporary increase in the rate of water loss, which is greater, the higher the saturation deficiency. However, after 3 or 4 days, the normal rate of dehydration is restored. This observation indicates that the hypodermis of the pupa is able to restore the water impermeability of the cuticle after its impairment by treatment with peanut oil.

4. Successive daily treatments with peanut oil completely changes the permeability of the cuticle of the pupa so that, after two or three exposures, it becomes freely permeable to water. The rate of weight loss is then roughly proportional to the saturation deficiency of the air. It seems likely that the oil brings about its effect by dissolving a fatty or waxy substance from the surface of the cuticle.

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SENSITIZATION OF THE FROG OVARY FOLLOWING HYPOPHYSECTOMY[1]

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THE production of ovulation in *Rana pipiens* by the injection of frog pituitary glands has become a common laboratory procedure for obtaining living embryological material. During the spring months of March and April the injection of one anterior lobe will effect 75–100 per cent ovulation, but in November this dosage brings about no ovulation whatsoever; ordinarily, six to eight glands are required to produce complete ovulation at this time. Rugh (1939) has shown that, if the anterior lobe is removed shortly before the pituitary material is administered, the dosage necessary to induce a given amount of ovulation is markedly reduced. He found that in November an average of 52 per cent ovulation resulted when hypophysectomized females were injected with one anterior lobe. Rugh therefore suggested that the frog's own pituitary

[1] Aided in part by a grant from the Milton Fund, Harvard University, administered by Professor F. L. Hisaw.

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gland may exert an inhibitory influence on the ovulatory reaction and that this inhibition is removed when the animal is hypophysectomized.

It seemed important to make a more thorough study of the effect of hypophysectomy on pituitary-induced ovulation *in vivo* and also to investigate the effect of hypophysectomy on the capacity of the ovary to respond to frog pituitary substances *in vitro*. Large, sexually mature, female frogs (50–100 gm.), obtained directly from hibernation, were used in all experiments. After arrival in the laboratory, they were stored until needed in a cold room at 4°.5 C., since ordinary room temperatures of 20°–25° C. were found to decrease the responsiveness of the ovarian tissue of normal frogs to pituitary substances. The smaller frogs (50–70 gm.) were suitable for all experiments which were carried out entirely *in vivo*, but larger females (70–100 gm.) were required when ovaries were to be excised for the study of response *in vitro*. Frogs under light ether anesthesia were hypophysectomized through the roof of the mouth by drilling a hole in the basisphenoid bone with a 2-mm. trephine and sucking the anterior lobe out into a pipette. Ordinarily, very little bleeding took place, and the frogs usually recovered from the effects of the anesthetic within 20 minutes.

The technique used for *in vitro* experiments has been described in detail in a recent paper (Wright, 1945). In brief, ovaries were cut into small bits (i.e., fragments containing 15–30 eggs), and these were suspended by cotton thread in stoppered vials, each containing the equivalent of one-sixteenth of an anterior pituitary gland from an adult female triturated in 10 cc. of Holtfreter's fluid. In each experiment conducted on the tissue of a given ovary eight such vials were prepared. The percentage of ovulation recorded for each test thus represents an average of the values obtained in the eight preparations. Controls were always included in which bits of the ovaries were suspended in Holtfreter's fluid, but in none of these did ovulation occur

In estimating percentages of ovulation *in vivo*, the number of eggs ovulated (i.e., in the body cavity, oviducts, or uteri) was compared with the number of eggs still remaining in the ovary, and thus the total present in the ovaries before ovulation started was readily determined. Percentages of ovulation *in vitro* were calculated (28 hours after the experiments were started) by dividing the number of eggs ovulated (i.e., fallen to the bottoms of the vials) by the total number of eggs in the ovarian fragments before ovulation began.

EXPERIMENTAL PROCEDURE AND RESULTS

All experiments described were carried out during the last week of December and the month of January. In order to obtain a base line for the interpretation of experimental results, the response of ten normal frogs (av. wt. 62.7 gm.) to the injection of the equivalent of one female frog pituitary gland was determined during the second week in January. Two of these animals did not ovulate; the average percentage of ovulation, 32 hours after injection of all ten animals, was 31.0 per cent. The wide variation in response (0–45 per cent) to identical doses of pituitary material is not abnormal for this time of year.

A group of fourteen animals (av. wt. 61.9 gm.) were hypophysectomized and, after remaining at room temperature for lengths of time of from 20 minutes up to 6½ days (from the time of hypophysectomy), were injected with the equivalent of one pituitary gland from a stock sus-

pension in Holtfreter's fluid. Thirty-two hours after injection the percentages of ovulation were determined. The results (Fig. 1) show a marked increase in the response of the ovary after hypophysectomy and are in agreement with data previously reported by Rugh (1939).

As controls, eight animals were subjected to sham operations, i.e., a hole bored in the basisphenoid bone, meninges pierced, but the anterior lobe left in place. Sixteen hours after the operation these females were given the equivalent of one pituitary gland, and 32 hours later all had ovulated 100 per cent of their eggs. Four females were decerebrated (and consequently hypophysectomized) by a quick slash of sharp scissors. None of these animals ovulated after the injection of one pituitary gland. However, it should be pointed out that considerable blood is unavoidably lost as a result of such drastic procedure, and this may be an important factor contributing to the negative response.

Similarly, a group of ten animals were hypophysectomized and returned to a cold room immediately after the operation. After remaining at 4°.5 C. for various lengths of time (from 16 hours to 9 days), the frogs were again removed to room temperature and injected with the equivalent of one female pituitary gland.

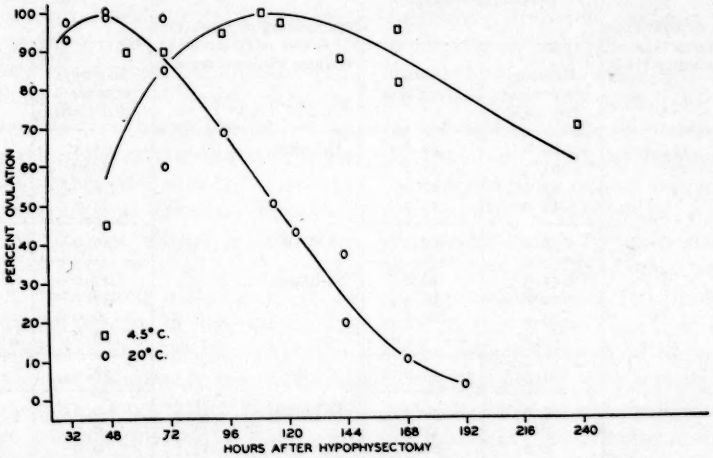

FIG. 1.—Ovulation in twenty-four hypophysectomized frogs (av. wt. 61.9 gm.) in response to the injection of the equivalent of one anterior pituitary gland. Ten frogs were returned to a cold room for varying intervals following hypophysectomy, while fourteen frogs were kept at laboratory temperatures. Two animals were injected immediately after hypophysectomy. In all frogs, 32 hours were allowed for ovulation to take place after the injection of the pituitary material. Ten normal animals (av. wt. 62.7 gm.) ovulated an average of 31.0 per cent after receiving equivalent doses of pituitary glands. Experiments conducted December 27 to January 31.

The results for these experiments (Fig. 1) seem to show the same increased responsiveness as that observed in experimental animals kept at room temperature. It is important, however, to note that the effect of hypophysectomy does not become evident immediately in frogs returned to the cold. Similarly, the sensitizing effect does not disappear so rapidly in animals kept at low temperatures.

To test the effect of hypophysectomy on the capacity of the ovary to respond to pituitary factors *in vitro*, ovarian

tissue was taken from twelve large hypophysectomized frogs which had been kept at room temperature after the operation. If pieces of the ovaries were removed 16 hours after hypophysectomy and placed in pituitary suspensions, normal frogs. However, when hypophysectomized females remained at room temperature for 72 hours, fragments of the ovaries failed to respond to pituitary suspensions. After normal frogs had remained at room temperature for 72

TABLE 1*

RESULTS OF *in vitro* EXPERIMENTS

Percentages of Ovulation *in vitro* in Pieces of Ovaries Removed after—	Uninjected Animals		Percentages of Ovulation *in vitro* in Pieces of Ovaries Removed after—	Injected Animals	
	Hypophysectomized (Per Cent)	Control (Per Cent)		Hypophysectomized (Per Cent)	Control (Per Cent)
16 hours	69.6	52.6	32 hours	91.3	52.9
	61.3	32.2		85.6	49.1
	53.9	27.4		75.0	44.6
	48.0	23.7		71.7	.....
Av.	58.2	34.0	Av.	80.9	48.9
48 hours	53.3	46.0	64 hours	47.0	50.0
	32.8	38.4		37.5	.....
	29.3	22.5		36.0	.....
	21.9	.....		33.6	.....
Av.	34.3	35.6	Av.	38.5	.....
72 hours	0.0	36.3	88 hours	0.0	57.5
	0.0	31.7		0.0	51.1
	0.0	22.1		0.0	43.0
	0.0	.....		.....	41.5
	.....	.....		.....	30.1
Av.	0.0	30.0	Av.	0.0	44.6

* In these experiments, experimental and control frogs were removed from the cold-room supplies at the same time.

Uninjected animals.—Experimentals were hypophysectomized immediately and, together with the controls, remained at room temperature for 16, 48, or 72 hours, after which pieces of the ovaries were removed for testing *in vitro*.

Injected animals.—Procedure as above except that after 16, 48, or 72 hours, frogs were injected with the equivalent of one pituitary gland from adult female frogs. An additional 16 hours elapsed after injection, and then pieces of the ovaries were removed for *in vitro* tests.

The concentration of triturated pituitary material in all *in vitro* preparations was the equivalent of one-sixteenth of an anterior lobe (from adult female frogs) per 10 cc. of Holtfreter's solution. All experiments were performed in January on ovarian tissue from 42 frogs.

greater percentages of ovulation were obtained (av., 58.2 per cent, Table 1) than in ovarian fragments from normal animals (av., 34.0 per cent) which had remained at room temperature for the same length of time. If the ovaries were removed 48 hours after hypophysectomy, about the same percentages of ovulation (Table 1) were achieved as in control vials containing ovarian tissue of hours, their ovaries did not show this refractoriness to pituitary factors *in vitro*, and 30 per cent ovulation (Table 1) was still obtainable. Apparently, then, in the absence of the pituitary gland the ovary undergoes physiological changes, even in 3 days' time, such that it is incapable of responding *in vitro*, although a response *in vivo* can still be obtained 6 days after hypophysectomy.

In a previous paper (Wright, 1945) it was shown that the injection of one pituitary gland into an intact frog brought about a sensitization of the ovary such that ovarian fragments responded more readily to pituitary suspensions than those removed from normal, uninjected animals. It seemed important, therefore, to investigate the effect of hypophysectomy on the capacity of the ovary to become sensitized after receiving a pituitary injection. If a frog was hypophysectomized, kept for 16 hours at room temperature, injected with one female pituitary gland, and the ovary removed 16 hours thereafter, an average of 80.9 per cent ovulation (Table 1) was induced *in vitro* with one-sixteenth of a pituitary gland. Ovarian fragments of normal frogs sensitized in this way ovulated an average of 48.9 per cent in pituitary suspensions identical to those used on the ovaries of hypophysectomized animals. If the hypophysectomized frogs were allowed to remain for 48 hours at room temperature before the injection of one pituitary gland, an average of only 38.5 per cent ovulation (Table 1) was obtained. If this period was extended to 72 hours, no ovulation was induced *in vitro*. Ovarian tissue from normal animals sensitized after the frogs had remained at room temperature for 3 days still ovulated an average of 44.6 per cent (Table 1). It is quite apparent, then, that the increased responsiveness of the ovary brought about as a result of hypophysectomy can be accentuated by the injection of pituitary material.

DISCUSSION

The results presented above do not seem to support the contention (Rugh, 1939) that an ovulatory inhibitor is removed when the frog is hypophysectomized. Since control animals, in which sham operations were performed and the anterior lobe left in place, showed the same increased responsiveness as that exhibited by hypophysectomized females, it seems more tenable to propose that in the process of hypophysectomy an ovarian sensitizer is released from the anterior lobe and into the blood stream. This release may be brought about by drilling with the trephine, by suction on the gland itself, or by any combination of the processes involved in hypophysectomy. Indirect evidence for this hypothesis is found in the fact that females show for several days after hypophysectomy the dark color reaction caused by the release of intermedin from the pituitary gland. If the procedure of hypophysectomy can set free a considerable quantity of intermedin, it is similarly possible that a hypophyseal gonadotropic substance may also be released during the operation. It is interesting to note in this connection that Lane and Greep (1935) attribute a temporary rise in ovarian weight, 72–96 hours after hypophysectomy in the immature rat, to the release of gonadotropic material from the pituitary gland into the blood stream during the operation. Williams (1945), however, does not agree that gonadotropins could be released at the time of hypophysectomy as the operation is performed in his laboratory.

Apparently, this hypophyseal factor set free during the manipulation of hypophysectomy brings about a rapid sensitization of the ovary, as evidenced by the response of the ovary both *in vivo* (Fig. 1) and *in vitro* (Table 1). This increase in ovarian sensitivity becomes maximal, at room temperature, at approximately 16 hours after the operation, after which it declines; and *in vivo* tests on the hypophysectomized female become negative by the seventh day. In contrast with this, ovarian fragments removed for *in vitro* experiments, 72 hours

after the operation, fail to respond to pituitary suspensions.

If the animal's metabolism is decreased by low temperatures, the full effect of this sensitization is postponed for some days. The refractoriness which appeared so rapidly at room temperature is also delayed, as shown by the fact that 70 per cent ovulation can still be obtained *in vivo* in response to the injection of one pituitary gland, 9 days after hypophysectomy. Judging by these results, it is probable that ovulation *in vitro* could be obtained for a longer time after ablation of the pituitary gland if the frogs were kept in the cold following hypophysectomy.

The identification of the sensitizing factor must await further investigation; however, results of recent experiments (unpublished) with the follicle-stimulating hormone (FSH) on ovulation in the frog indicate that the sensitizing agent released at hypophysectomy may be FSH. This hypothesis seems plausible in view of the fact that hypophysectomy seems to bring about only a sensitization of the ovary. So far as is known, no frog has ovulated spontaneously as a result of the mechanics of hypophysectomy, and further work is necessary to establish the nature of pituitary factors requisite for ovulation.

SUMMARY

The results of these experiments indicate that the process of hypophysectomy brings about a sensitization of the frog's ovary so that it responds more readily than normal to the injection of pituitary substances. These findings are in agreement with data previously reported by Rugh. In hypophysectomized females, 100 per cent ovulation occurred after the injection of one pituitary gland, while in normal animals this dosage produced an average of 31 per cent ovulation. Control animals in which sham operations were performed also ovulated 100 per cent of their eggs after receiving this same dosage of pituitary material.

Hypophysectomy also seemed to bring about an increased responsiveness of the ovary to pituitary substances *in vitro;* this sensitivity could be accentuated by implanting a pituitary gland some hours before removing the ovary for experimentation. The responsiveness of the ovary both *in vivo* and *in vitro* was maximal at about 16 hours after hypophysectomy (at 20° C.); but, by 72 hours after the operation, ovarian fragments did not respond *in vitro;* and *in vivo* tests, 7 days after hypophysectomy, were negative.

When hypophysectomized females were returned to a cold room, the full effect of sensitization was not apparent until about the third day. On the other hand, the ovaries did not become rapidly refractory as did the ovaries of animals kept at room temperature, and 70 per cent ovulation was obtained *in vivo* 9 days after hypophysectomy.

The temporary sensitization which follows hypophysectomy is thought to be brought about by the release of a sensitizing factor from the anterior lobe during the course of the operation.

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OXYGEN CONSUMPTION OF LAMPREYS, WITH SPECIAL REFERENCE TO METAMORPHOSIS AND PHYLOGENETIC POSITION

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ALTHOUGH a considerable literature has accumulated on the normal oxygen requirements of numerous representatives of chordate animals, a conspicuous absence of information occurs for the most primitive of the vertebrate series, the cyclostomes (Agnatha). Horton (1932), in a paper principally describing unsuccessful attempts to induce metamorphosis in larval lampreys (ammocoetes), states that thyroid preparations are effective neither on metamorphosis nor on metabolism of *Lampetra* material. But Horton gives none of the actual data upon which the reference to metabolism was based. The writer has likewise made general statements concerning the metabolism of lampreys (Leach, 1939, 1944) in connection with morphologic and phylogenetic studies, without presenting his specific data on oxygen consumption.

The present report deals mostly with the oxygen consumption of full-grown ammocoetes of the brook lamprey, *Ichthyomyzon fossor*, and their normal metabolism during metamorphosis. Less extensive studies are also reported here on the adults of *I. fossor* and on full-grown ammocoetes and juvenile lampreys of the lake form, *Petromyzon marinus* (land-locked). Many of the present data were obtained in connection with morphological studies of the endostylar organ and its transformation into definitive thyroid follicles. Therefore, some emphasis has been placed on the metabolic rates of lampreys with reference to the delayed and unique transformation of the thyroid complex and with an apparent arrested-growth period in the life-history.

In view of the conditions stated above and the circumstance of relatively difficult and limited material, no attempt has been made to follow modern statistical methods in presenting results. However, the data presented are readily comparable with a review prepared and tabulated by Heilbrunn (1943) on the metabolic rates of various chordates representing all classes except for Cyclostomata. The material presented here would, therefore, appear to be of general biologic interest, in that it serves to fill a striking gap when one considers Heilbrunn's review phylogenetically.

MATERIALS AND METHODS

All the *Ichthyomyzon* material used in the present report was collected from the Tippecanoe River near Warsaw, Indiana, mostly during the month of June. The ammocoetes used were regarded as "full grown," i.e., they were within the range of transformation size before the onset of a transformation season. My previous study (1940) of the life-history of this species shows, however, that only about one-third of the individuals of this range do transform at the earliest transformation season and that many of the largest individuals to be found do not transform at the season when one would expect them to do so. *Ichthyomyzon fossor* is a nonpredatory form, in which adults become sexually ripe a few months after metamorphosis.

The lake lamprey ammocoetes were taken from the Salmon River near Pulaski, New York. My observations (unpublished) on the lake lamprey indicate that about two-thirds of the "full-grown" ammocoetes transform at the earliest transformation season; but, as was first pointed out by Gage (1927), some of the largest ammocoetes of this species also spend an entire year as "maximum-size" ammocoetes. The onset of the transformation season for both species is from mid-August to mid-September. Nothing is definitely known as to the exact number of years that any of the lampreys spend as ammocoetes, but possibly the larvae of all species spend one full year in an "arrested-growth period" preparatory to general metamorphosis.

For successful culture, the writer attaches considerable importance to keeping the burrowing ammocoetes in some of the stream deposits from which they are taken. Consequently, a quantity of this debris is collected with the animals. During experimental work, however, and when they are being transported over long distances, they are best kept in clean washed sand taken from stream beds in which smaller ammocoetes are usually found. The larger ammocoetes are usually found in stream deposits containing considerable mud and organic matter; but, if they are obliged to swim freely in muddy water, they soon develop respiratory difficulties. For this work individual ammocoetes were kept in gallon battery jars about one-third full of washed sand covered with 2 or 3 inches of water. Even large ammocoetes may be kept for a month or more in well-washed sand with no apparent ill-effects. In general, ammocoetes were returned at suitable intervals to material containing a richer supply of diatoms and other small algae upon which they feed. The burrowing animals may be easily removed from the jars by pouring off the water and digging the sand gently with the fingers until they wriggle from their burrows. Care must be used in handling the specimens to prevent injury, but they are surprisingly hardy experimental animals.

Measurements were made by allowing the animal to wriggle into a culture dish containing a few cubic centimeters of water. When the dish is held at a slight angle and the animal's body is allowed to come into close contact with a small ruler, it will usually remain quiet long enough to permit an accurate reading to be made. Weights were determined by causing the animal to wriggle into a screen-wire sieve, from which it was transferred into a culture dish of water, previously counterbalanced with another similar dish. Since the specific gravity of the animal is only slightly greater than water, some checks on accuracy of weights were made by permitting the animal to enter a small-bore burette containing water and then reading the displacement.

For determining oxygen consumption, the Winkler sealed-jar method was used. Animal test bottles were of 1-liter capacity; sample bottles, 100 cc.; and the test periods were almost always of 3-hour duration in semidarkness. The water supply was from a shallow well and was well aerated. Temperatures were kept constant by immersing the bottles containing the animals in a rather large tank of water, previously brought to the required temperature. Usually ten animals were tested concurrently but individually. When both species were tested for comparative purposes, five representatives of each species were used concurrently. After some initial restlessness, the animals usually lie quietly throughout the period. However, it should be noted that the conditions described create an environment that is somewhat unnatural to the

ammocoete, which normally leaves its burrow only when disturbed. A valuable discussion of the Winkler method applied to metabolism studies is given by Allee and Oesting (1934).

OXYGEN CONSUMPTION OF LARGE AMMOCOETES

Each of the five groups in Table 1 consists of five individuals of similar size, but not necessarily of the same age. From my own studies of these two species, all individuals of all these particular groups are within the size range of metamorphosis (during August and September), and both maximum and minimum lengths are represented for the known range of metamorphosing individuals. The oxygen consumption in all cases is expressed in cubic centimeters per gram weight of live individuals per hour. The data of Table 1 indicate that the oxygen consumption of the ammocoetes of the lake lamprey is consistently higher than that of the brook form and that the difference is relatively greater in the groups of smaller individuals. These differences may be partly due to the more slender form of the lake lamprey and the possibility that more individuals of the groups of smaller animals in this form belong to a younger age group than do individuals of similar size belonging to *I. fossor*. Although it is impossible to determine the exact age of large ammocoetes, all in Table 1 are almost certainly at least four years old.

TABLE 1

OXYGEN CONSUMPTION OF LARGE AMMOCOETES IN JUNE

(Water Temperature 18° C.)

I. fossor				*P. marinus* (LAND-LOCKED)			
Group No.	Av. Length (Mm.)	Av. Wt. (Gm.)	O_2 (Cc/Gm/Hr)	Group No.	Av. Length (Mm.)	Av. Wt. (Gm.)	O_2 (Cc/Gm/Hr)
1	148	5.1	0.103	1	156	5.4	0.118
2	137	4.2	0.101	2	145	4.6	0.111
3	131	3.8	0.107	3	137	3.8	0.124
4	126	3.1	0.108	4	130	3.0	0.142
5	121	2.7	0.118	5	124	2.6	0.152

OXYGEN CONSUMPTION OF *I. fossor* DURING EARLY METAMORPHOSIS

The material of this study was collected from the stream on August 27, when only two of a collection of thirty-two full-grown ammocoetes exhibited external metamorphic change. Studies on oxygen consumption were begun 4 days later. In all, thirty individuals were tested on September 5; but, of these, only eight subsequently transformed. The oxygen consumption of these is given in Table 2, with comparative data for eight nontransforming ammocoetes most closely matched with metamorphosing individuals in length and weight. Subsequent comparative data for some of these individuals in Table 2 are given in Figure 1.

The results shown in Table 2 are not what might be expected when one considers the rapidity and the extent of cellular changes and the reorganization of tissues and organs that occur during this period. These internal activities are well advanced before external evidences of metamorphosis make their appearance. The changes strikingly affect the respiratory tissue and endostyle, which become extremely vascular and undergo extensive histolytic changes which last several

days. The details of this phase are described by Leach (1939), and representative conditions of the endostyle are reproduced here in Figure 1.

One might presume that such profound internal activity in transforming individuals would call for a marked increase in oxygen requirements. At two previous transformation seasons the writer had attempted to determine which ammocoetes of a collection of full-grown individuals were undergoing early metamorphosis. Metabolic tests were initially made with the view of selecting early stages of metamorphosis for morphologic studies without killing the animals. These attempts were all futile, and I know of no way, except by histologic examination, to distinguish between the large ammocoetes destined to transform at a particular season and those that will not. My studies have consistently shown that no increase in oxygen consumption accompanies the early stages of metamorphosis and strongly indicate that an actual decrease in metabolic rate occurs frequently, if not usually, during these stages, as determined by oxygen-consumption tests.

TABLE 2*

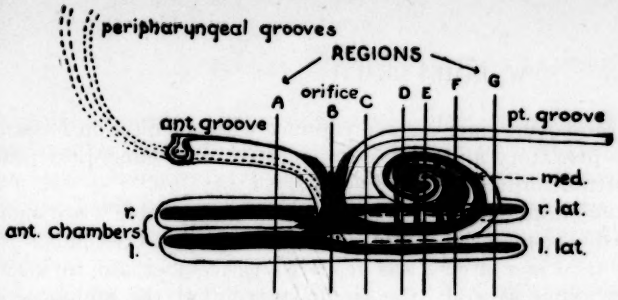
OXYGEN CONSUMPTION OF NONTRANSFORMING AMMOCOETES OF *I. fossor* COMPARED WITH MATCHED TRANSFORMING INDIVIDUALS

Group	Length of Individuals (Mm.)	Sept. 5	Sept. 9	Sept. 15	O_2/Cc/Gm/Hr 18° C.		
					Sept. 5	Sept. 9	Sept. 15
1	152	−	−	−	0.140	0.136	0.156
	148	+	+	++	0.102	0.094	0.118
2	140	−	−	−	0.147	0.126	0.142
	140	?	+	+	0.142	0.114	0.127
3	136	−	−	−	0.156	0.173	0.182
	138	−	−	+	0.127	0.129	0.131
4	135	−	−	−	0.156	0.162	0.151
	135	?	+	++	0.140	0.128	0.161
5	131	−	−	−	0.128	0.142	0.151
	132	+	++	++	0.127	0.131	0.145
6	125	−	−	−	0.138	0.142	0.150
	121	?	+	++	0.172	0.168	0.210
7	120	−	−	−	0.157	0.182	0.147
	120	++	++	++	0.119	0.136	0.160
8	118	−	−	−	0.196	0.174	0.132
	118	+	+	++	0.120	0.118	0.132
Av. for 8 transforming individuals					0.131	0.127	0.148
Av. for 8 nontransforming individuals					0.153	0.154	0.150

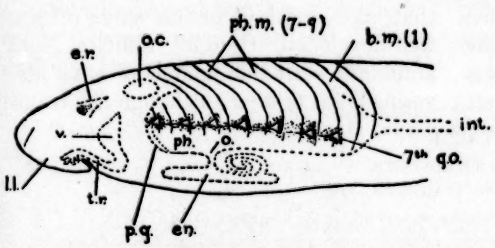
* Transformation changes: −, none; ?, doubtful; +, slight; ++, distinct.

AMMOCOETES AND YOUNG ADULTS OF *P. marinus*

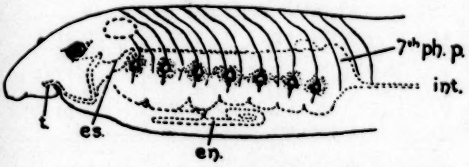
The lake lamprey material was limited, and no attempt was made to secure comprehensive data on the oxygen con-



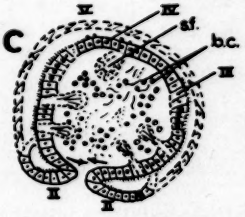
ENDOSTYLE OF GROWING AMMOCOETE

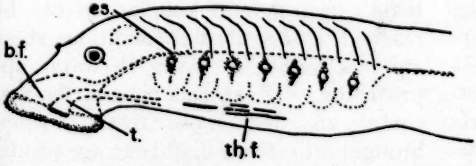


AMMOCOETE GROWTH PERIOD



EARLY TRANSFORMATION STAGE





ACTIVE ADULT PERIOD

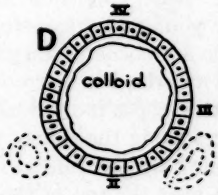


FIG. 1.—Metamorphosis of the thyroid complex of *I. fossor* with reference to general transformation changes of the animal. Endostylar chambers of a growing ammocoete are shown in solid black. *A*, distribution of epithelial cells of endostyle in the left anterior chamber (region *A*); *B*, differentiation of semi-follicles during arrested-growth period; *C*, histolytic phase of endostylar transformation at early metamorphosis, showing fate of semi-follicles, etc.; *D*, definitive thyroid follicle of adult: *a.c.t.*, areolar connective tissue; *b.c.*, blood cells; *b.f.*, buccal funnel; *b.m.* body myotome; *d.l.* and *d.m.*, dorsolateral and dorsomedial columns; *en.*, endostyle; *e.r.*, eye rudiment; *es.*, esophagus; *g.o.*, gill opening; *int.*, intestine; *o.c.*, otic capsule; *ph.*, pharynx; *p.g.*, peripharyngeal grooves; *s.f.*, fate of semi-follicles; *t.*, tongue; *th.f.*, thyroid follicles; *t.r.*, tongue rudiment; *I–V*, types of endostylar cells. Modified and adapted after Leach (1939, 1940).

sumption of this form. Moreover, since adults of this species are predatory and pass through a posttransformation growth period, strictly comparable data with *I. fossor* are precluded. The material used in Table 3 had been kept in a large greenhouse aquarium containing several species of fish; but, up to the time the tests were run, the metamorphosing individuals had not been seen to attack them. They did so later, however. When tested, the juvenile lampreys were in the macrophthalmia stage of metamorphosis supposed to be associated with the onset of the predatory period. Tests were run at the temperature of the water in the greenhouse significantly higher than temperatures for *I. fossor* (see Tables 2–3). One other condition should be noted. The young adults, instead of burrowing, as do ammocoetes, merely hide in aquarium debris. Furthermore, while in test bottles, these individuals suspend themselves along the side of the bottle by attaching by the buccal funnel at a position near the top of the bottle. I do not know what effect, if any, this change of position in the test has on the oxygen consumption; but one might infer that more energy would be required to suck on the side of the bottle than to lie quietly on the bottom. The results do not agree with the inference, however, and are at some variance with the data on *I. fossor* of approximately the same posttransformation age.

The data shown in Table 3 are again surprising. The first test (December 10) was repeated (December 26) to verify the circumstance that the ammocoetes appeared to have a metabolic rate approximately 50 per cent higher than young adults. Marine (1913) has shown that lake lampreys in this stage possess well-developed thyroid follicles with abundant stainable colloid. I have also verified this finding. Although the transformed individuals were consistent in showing a lower oxygen consumption, the writer feels that this period should be studied with more individuals and at a lower water temperature than employed here, before definite conclusions can be reached. Under normal conditions these animals would be living in water approaching 0° C. at this season. Ammocoetes are also more restless when brought into strong light than are adults. Furthermore, definitive follicular thyroid tissue may operate differently under different conditions of temperature.

TABLE 3

OXYGEN CONSUMPTION OF AMMOCOETES AND YOUNG ADULTS OF *P. marinus* (LAND-LOCKED)

(Averages Given on Tests Made December 10 and 26, 1943; Temperature 22°.5 C.)

Ammocoetes				Transforming Specimens			
Individual	Length (Mm.)	Weight (Gm.)	O_2/Cc/Gm/Hr	Individual	Length (Mm.)	Weight (Gm.)	O_2/Cc/Gm/Hr
1	142	4.2	0.36	1	152	5.3	0.21
2	140	4.1	0.41	2	142	4.0	0.26
3	137	3.8	0.29	3	131	3.1	0.17
4	124	2.6	0.45	4	124	2.7	0.18
5	120	2.5	0.39	5	122	2.6	0.32

SEMISEDENTARY AND ACTIVE ADULT PERIODS

Data for these periods are shown in Figure 2, which also gives comparative

oxygen-consumption curves of matched ammocoetes. The individuals used here were selected representatives of specimens in Table 2. These data will be reviewed in the following general discussion.

GENERAL DISCUSSION

The circumstance that no increase in oxygen consumption accompanies early metamorphosis and that a significant decrease may occur would at least indicate that new factors having a stimulating effect on metabolism are either not produced by the new thyroid complex or not released under my laboratory conditions. The endostyle would appear as effective as the follicular thyroid so far as maintaining the metabolic rate in relatively inactive individuals. I have conducted numerous experiments with various iodine and thyroid preparations on all stages of lampreys without producing positive effects either on oxygen consumption or on metamorphosis.

Under normal conditions one may as-

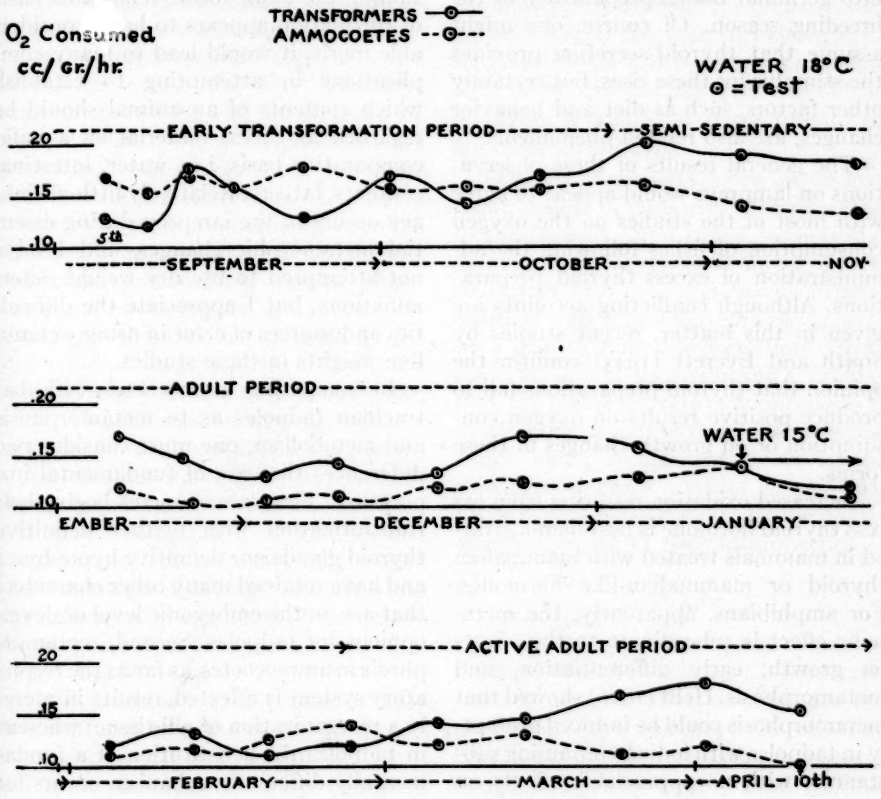


FIG. 2.—Oxygen consumption of *I. fossor*. These curves show the consumption based on the average for five transforming individuals compared with the average for five ammocoetes, which were matched in size at the onset of the transformation season. Weight determinations were made monthly, and changes in weight were spread over the test periods during which they occurred. Note that the tests were conducted at lower water temperature after the middle of January. Weight reduction for transforming individuals was most pronounced during October and March.

sume that two periods occur in the adult life of *I. fossor* when oxygen requirements may be greater than for large ammocoetes of this form: first, immediately following the conclusion of metamorphosis and, second, at the period of rapid sexual ripening. The first rise may be ascribed to the inability of the animal to feed during and following transformation, thus changing the internal nutritional conditions. The second rise may represent an acceleration of expenditure of energy in transforming somatic tissue into germinal tissue, preparatory to the breeding season. Of course, one might assume that thyroid secretion provides the stimulus for these rises, but certainly other factors, such as diet and behavior changes, are also related phenomena.

The general results of these observations on lampreys would appear to agree with most of the studies on the oxygen consumption of fishes following the administration of excess thyroid preparations. Although conflicting accounts are given in this matter, recent studies by Smith and Everett (1943) confirm the opinion that thyroid preparations fail to produce positive results on oxygen consumption or on growth changes in these forms.

Increased oxidation resulting from excess thyroid hormone is best demonstrated in mammals treated with mammalian thyroid or mammalian-like hormones. For amphibians, apparently, the metabolic effect is subordinate to the effects on growth, early differentiation, and metamorphosis. Helff (1926) showed that metamorphosis could be induced promptly in tadpoles with iodine-containing substances, with no appreciable effects on oxygen consumption. He regarded the metamorphic and metabolic factors in mammalian thyroid as independent properties of thyroxine.

Etkin (1934) questioned the view that oxygen consumption is increased in tadpoles during metamorphosis, and, in a later paper (1936), showed that the assumption that marked activity of the thyroid gland occurs in tadpoles undergoing normal metamorphosis is not supported by his histological studies. However, Etkin's (1934) conclusions that no increased metabolism accompanies metamorphosis in tadpoles is based on his dry-weight determinations, which show considerable constancy throughout the decrease in wet weight of individuals assuming the adult form. While this basis of calculation appears to have considerable merit, it would lead to many complications in attempting to establish which contents of an animal should be regarded as excess material on a valid comparative basis, i.e., water, intestinal contents, fat, etc. Relatively little shrinkage occurs in the lamprey during essential metamorphic changes, and I have not attempted to use dry-weight determinations; but I appreciate the difficulties and sources of error in using wet and live weights in these studies.

In comparing ammocoetes with batrachian tadpoles as to metamorphosis and metabolism, one must consider two differences that are of fundamental importance. First, ammocoetes begin their transformation with neither definitive thyroid glands nor definitive hypophyses and have retained many other characters that are on the embryonic level of development for tadpoles. Second, metamorphosis in ammocoetes, as far as the respiratory system is affected, results in merely a reorganization of gill tissue; whereas in tadpoles the substitution of a fundamentally different mechanism occurs for absorbing oxygen. The loss of other fish-like characters and habits is also striking.

The circumstance that the lamprey does not lose weight rapidly during metamorphosis affords a more satisfactory

condition for a study of oxygen consumption than do tadpoles, in which shrinkage is extremely rapid and weight calculations more subject to error. This is particularly evident during the critical period in tadpole metamorphosis when they are neither strictly aquatic nor strictly terrestrial. At this time it would appear that methods of testing might influence the results because of unnatural and forced conditions of existence and the possibility of the acquirement of an oxygen debt.

My morphological studies on the endostyle and thyroid gland of *I. fossor* (1939) indicated that the definitive gland in this animal represents the most primitive anatomical condition of the gland of all the vertebrates. From morphologic data the opinion was reached that the endostyle (before transformation) acted as a primitive thyroid mechanism concerned principally with growth and that a second property concerning metamorphosis was acquired during the ammocoete arrested-growth period as indicated by the formation of "semi-follicles," which are used up early in transformation (Fig. 1, *B*). The assumption was made that a property affecting metabolism, if present at all, was not dominantly active until the active adult period was reached (Fig. 1, *D*). This sequence of appearance and dominance of the properties was looked upon as progressively accumulative and phylogenetically significant. I have more recently elaborated on these points elsewhere (Leach, 1944).

Until Gorbman and Creaser (1942) demonstrated that the endostyle of ammocoetes was capable of iodine accumulation and storage, nothing was definitely known regarding its function. In my interpretation of the endostyle (1939), the organ was regarded as a primitive pouch, designed to take in and circulate water, from which dissolved substances could be absorbed and from which elements (except food) could be mixed with mucus and delivered to the esophagus. The endostyle of ammocoetes is definitely not analogous with the endostylar apparatus of acraniates and also is not strictly homologous with them.

Gorbman (1941) showed that the endostyle of an ascidian was incapable of iodine accumulation and, in his later paper (with Creaser, 1942), reported that the endostyle of amphioxus is likewise incapable of iodine accumulation. Thus it can now be stated that the endostyle of ammocoetes is the most primitive thyroid mechanism which possesses a chemically functional analogy with the follicular type of gland so characteristic in adults of all craniates.

The data presented here on the oxygen requirements of lampreys with reference to the thyroid gland would support the opinion that the gland is not so much concerned with the regulation of metabolism of the lower vertebrates as it is with growth and differentiation. However, according to Heilbrunn's tabulation, the metabolic rate of ammocoetes is most nearly similar to that of some fishes, which under similar conditions is approximately twice that of amphioxus. Little is yet known concerning the physiologic evolution of thyroid properties.

SUMMARY

1. The oxygen consumption of ammocoetes of the brook lamprey, *I. fossor*, appears to be somewhat less than for the predatory lake lamprey, *P. marinus* (land-locked). The ratio is approximately 0.10 cc/O_2/gm/hr to 0.125 at 18° C. (live weight).

2. No increase in oxygen consumption accompanies the normal metamorphosis of *I. fossor*, and some decrease is indicated in critical phases of the process.

3. In *I. fossor*, rises in oxygen consumption appear (1) in the semisedentary period sometime after colloid appears in the definitive thyroid follicles and (2) in the active adult period preparatory to the ripening of the gonads. These rises are not to be regarded as necessarily indicating an increased thyroid activity.

4. Results of this study indicate that the thyroid gland in lampreys is not concerned with metabolism in the manner described for amphibians and the higher vertebrates.

5. The metabolic rate for larval and adult lampreys is more nearly similar to fishes than to amphioxus. Since the endostyle of ammocoetes is now known to store iodine, this similarity might be expected.

6. Administration of thyroid substances has no effect on either metamorphosis or metabolism in lampreys.

7. This review supports a hypothesis previously proposed, that properties of thyroid secretion in higher vertebrates have developed phylogenetically with a progressive sequence of dominance affecting growth, metamorphosis, and metabolism.

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PIGMENTATION STUDIES IN SALAMANDERS, WITH ESPECIAL REFERENCE TO THE CHANGES AT METAMORPHOSIS[1]

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ALTHOUGH the amphibian pigmentary pattern changes conspicuously at metamorphosis, few investigators have attempted to describe the developmental processes which take place. Most of the literature contains only brief descriptions of the gross changes which are visible externally (Banta and McAtee, 1906; Uhlenhuth, 1916, 1917; Grant, 1930). Those who have studied the histological aspects of the problem have described only the typical embryo, larva, and adult and have made no attempt to show the sequence of events which leads to the definitive pigmentary pattern and the complete development of the dermis (Dawson, 1920; Wilder, 1925; Berweger, 1926; Elias, 1931, 1934). Three types of pigment cells are commonly found in the larval and adult stages of amphibians and have been noted by many authors (Weidenreich, 1912; Fuchs, 1914; Biedermann, 1926, 1928, 1930). They are (1) the lipophores or xanthophores, which contain a red or yellow pigment; (2) the guanophores, which contain highly refractile white or yellow crystals; and (3) the melanophores, which contain dark-brown or black pigment granules. The present investigation is primarily concerned with the melanophores which occur in the skin, although this type of pigment cell is widely distributed in the body of amphibians.

The theories which have been advanced concerning the origin of vertebrate pigment cells have been reviewed by Fuchs (1914) and Biedermann (1926, 1928, 1930) and, more recently, by DuShane (1943, 1944). At present the origin of melanophores from the neural crest in amphibians and birds has been so well established that other hypotheses no longer need to be considered. Until the last decade it was generally accepted that the dermis was derived from the mesoderm. More recently some investigators have suggested that the neural crest plays at least some part in dermal development (Raven, 1931, 1936; Holtfreter, 1935; Detwiler, 1937, 1938). Their evidence is largely inconclusive, however, because the work has been confined, for the most part, to early developmental stages when the dermis is poorly differentiated.

The object of the present investigation is to describe the histological development of pigmentation in the metamorphosing urodele and to correlate this with the gross pigmentary changes which occur at this time. A series of animals from the just-hatched larva to the adult was included in the study when it became apparent that many of the important pigmentary changes occur in the larva. However, it was soon realized that the development of the pigmentary pat-

[1] This paper is a revision of a dissertation presented to the faculty of the University of Chicago in partial fulfilment of the requirements for the degree of Doctor of Philosophy. I wish to express my sincere gratitude to Professor G. P. DuShane for suggesting this problem and for his aid during the investigation.

tern could not be described without considering the history of the dermis. Xenoplastic neural-crest grafts were used to aid in the analysis of some of the factors which influence the pigmentary pattern. This method is of value in determining what part of the specific differences in this pattern is controlled by the host environment and what part by factors intrinsic to the cells themselves. The graft material has been of special value in determining the relation between epidermal melanophores and the melanin found in epidermal cells.

Materials and Methods

The animals used in this investigation are *Ambystoma tigrinum*, *Ambystoma maculatum*, and *Triturus torosus*. The specimens of *A. tigrinum* and *A. maculatum* were obtained from eggs collected in ponds in the Chicago region. Specimens of *T. torosus* were obtained from eggs shipped from Stanford University, through the courtesy of Professor V. C. Twitty. After hatching, the larvae were isolated in finger bowls and kept in well water, which was changed every 3 or 4 days. For 2 or 3 weeks the larvae were constantly supplied with white annelid worms of the genus *Enchytrea* to assure maximum feeding. Thereafter, the larvae and, later, the adults were fed once a day with strips of raw liver.

In all cases length was determined by measurement from snout to tip of tail. The entire animal was then fixed in formol-Zenker's solution for 24 hours. In the normal series the head was cut off behind the neck in the smaller animals, while in the larger ones a square of skin and underlying muscle was removed from the dorsal cervical region. These pieces were imbedded in paraffin, while the rest of the animal was stored in 70 per cent alcohol.

The neural-crest grafts, which were made by Professor DuShane, consisted of grafting pieces of trunk neural folds orthotopically between embryos of *A. maculatum* and *T. torosus* of stages 14–22. In the graft series the anterior half of the animal was imbedded, and sections were made in the region of the graft. All sections were cut at 10 μ and stained either in Heidenhain's modification of Mallory's connective-tissue stain or in hematoxylin eosin azur.

In order to determine whether the increase in cell number observed at certain periods of development was the result of increased mitoses or of cell migration from other regions, two colchicine series were set up. Larvae of *A. tigrinum* were placed in a solution of 20 mg. colchicine per 100 cc. well water for 29 hours, while larvae of *A. maculatum* were placed in a solution of 100 mg. per 100 cc. well water for the same length of time. These were the approximate concentrations used by Mills (1939) in her work on *A. jeffersonianum*. The mitotic index (number of mitoses per 100 cells) was obtained from a count of at least one thousand cells of each type.

To aid in analyzing changes in the cell types which may be important in the development of pigmentation and of the dermis, counts were made of a standard area (four 10-μ sections, three fields per section, with an 8-mm. objective). To reduce variation the counts were made in a corresponding region in all animals: the mid-dorsal area of the skin in the cervical region. To obtain a representative sample, every eighth section was counted. It is obvious that there is an inherent disadvantage in this method: the surface of the animal increases many fold, so that in the just-hatched larva the area counted represents a much larger portion of the total body surface than does an area of the same size in an adult. Since the area counted is small, the

sampling error may be large. Melanophores are difficult to count accurately because the nuclei are often obscured by the melanin granules.

Measurements of dermal thickness were obtained by means of an ocular micrometer. Each value given is the average of six measurements made over as much of the dorsal surface as the section covered. There is considerable variation in different regions; hence the values are only approximations. They are considered significant, however, since the increase in thickness which takes place during development is a very large one.

Development of Pigmentation and the Related Development of the Dermis

The arrangement of melanophores in the skin varies greatly among different species of urodeles. The following observations were made on three species representing two different families. Although basic similarities will be brought out, it will become apparent that many small differences are present, especially between different families.

Normal development

A series of animals from the young larva through metamorphosis to the adult stage was obtained for each of the three species studied. For *A. tigrinum*, the interval between each animal in the series is about 5 mm., while in *A. maculatum* and *T. torosus*, which are smaller and slower developing, the interval is about 3 mm. Although it is the metamorphic changes with which this investigation is most concerned, it is necessary to recognize the earlier changes during the larval period in order to appreciate that the metamorphic changes are only the climax of earlier development. In *T. torosus*, study was also made of the first appearance of melanophores in the embryo.

A. tigrinum and *A. maculatum*

The sequence of events in the development of the dermis and of the pigmentary pattern is very similar in these species, with only minor variations. Plate I, showing the development of the skin of *A. tigrinum*, can serve to indicate the development in *A. maculatum* as well. In the larva which has just hatched, melanophores are found in the loose connective tissue of the subdermal region and in the epidermis. Subdermal melanophores are closely applied to the overlying layer of collagenous fibers which comprises the dermis at this stage (see *A*, Pl. I). This layer of fibers is extremely thin—2–4 μ in thickness—and contains no cells. In addition to melanophores, stellate-shaped cells containing no pigment also occur in the subdermal region, their processes adherent to the overlying dermis. There is no morphological basis for distinguishing these cells from the undifferentiated mesenchyme cells of the deeper loose connective tissue. It will be shown later, however, that the mesenchyme-like cells immediately below the dermis appear to contribute to the formation of the dermis and that at least a part of them are potential pigment cells. For this reason these cells will be referred to as "unpigmented cells." This does not mean that all cells referred to as unpigmented will become chromatophores, for this is certainly not the case. It is used for want of a better term to indicate that these cells, because of their position, may have a different prospective potency than the cells which occur at random in the loose connective tissue. In making the counts, only those cells were included which were located adjacent to the dermis.

There is no significant increase in the

number of subdermal melanophores per unit area during the first half of the larval period (Table I, *A. tigrinum*, and Table 2, *A. maculatum*). There must be an increase in the total number, however, because the surface area has increased several fold during this time. The proportion of rounded cells increases so that very few expanded cells are found directly below the dermis by the mid-larval period. The increase in cell number can be correlated with an increase in the mitotic index (number of mitoses per 100 cells), which is highest during the

TABLE 1

RELATION OF THE NUMBER OF MELANOPHORES AND UNPIGMENTED CELLS PER UNIT AREA TO THE DEVELOPMENT OF THE LAYERS OF THE SKIN IN LARVAL AND ADULT *Ambystoma tigrinum*

Length (Mm.)	Description	Subdermis			Dermis				Epidermis	Total	
		Unpigmented Cells		Melanophores	Unpigmented Cells		Melanophores	Thickness (μ)	Melanophores	Unpigmented Cells	Melanophores
		Number	Mitotic Index		Number	Mitotic Index					
24	Larva	47	6.3	17	0	0	0	3	8	47	25
29	Larva	123	6.7	13	0	0	0	4	4	123	17
33	Larva	119	3.7	8	0	0	0	7	7	119	15
39	Larva	145	1.8	17	0	0	0	8	13	145	30
44	Larva	187	1.3	12	2	0	0	10	7	189	19
48	Larva	214	1.7	16	1	2.6	0	12	9	215	25
53	Larva	171	2.0	16	3	0.3	1	13	11	174	28
58	Larva	193	0.8	19	3	0.2	1	17	7	196	27
63	Larva	207	.....	9	5	.....	1	14	17	212	27
67	Larva	206	1.0	11	13	2.6	3	20	3	219	17
73	Larva	179	.....	2	123	.....	9	33	8	302	19
79	Larva	109	2.3	3	171	7.4	19	41	6	280	28
85	Larva	99	2.0	2	211	3.5	19	61	3	310	24
92	Larva	70	.....	1	215	.....	13	116	3	285	17
97	Metam. I	77	.....	0	225	.....	18	120	4	302	22
100	Metam. II	74	.....	2	207	.....	23	165	6	281	31
107	Metam. III	107	.....	0	268	.....	29	185	1	375	30
117	Adult I	55	.....	9	345	.....	29	307	5	400	43
135	Adult II	52	.....	10	367	.....	49	324	17	419	76

subdermal unpigmented cells, on the other hand, increase very rapidly until the mid-point in larval development (50 mm. in *A. tigrinum;* 33 mm. in *A. maculatum*). During this time the unpigmented cells appear stellate-shaped for the most part, with elongated processes extending for some distance below the dermis; but some are rounded in shape, with an eccentrically placed nucleus and more basophilic cytoplasm. However, early larval period. Tables 1 and 2 give the mitotic index for dermal as well as subdermal unpigmented cells. This was obtained on animals of lengths corresponding to those in the normal series. An occasional mitosis was observed in melanophores, but the rate is too low to be measured with any degree of accuracy.

While the subdermal unpigmented cells are increasing in number, the layer

of collagenous fibers, which constitutes the dermis, increases in thickness to about 12 μ. Although the subdermal unpigmented cells are, for the most part, rounded in shape at this time, one may commonly see one or two fine processes extending toward the dermis (see *B*, Pl. I). Some of these processes penetrate the fibrous layer and extend laterally between the fibers. The fibers of the dermis are now more loosely arranged, and the dermis is thicker. The first appearance of entire cells within the dermis occurs soon after this, and many cells may be found with part of the cell body within the dermis and part below it. Subdermal melanophores are frequently seen with processes extending into the dermis, and rarely the major part of the cell body occurs in the dermis, with only a single process extending from the subdermal region (see *C*, Pl. I).

In *A. maculatum* the appearance of cells in the dermis is paralleled by changes in the pigmentary pattern of the living animal. The larva assumes a darker appearance quite suddenly, and light spots appear along the flanks, indicating the position of the lateral-line organs. This change is a conspicuous one and was described by Uhlenhuth (1917) as the formation of the "network stage." No such change in external appearance has been noted in *A. tigrinum* to mark the first appearance of cells in the dermis.

In the more advanced larva there is a rapid increase in the number of melanophores and unpigmented cells in the dermis and, at the same time, a de-

TABLE 2

RELATION OF THE NUMBER OF MELANOPHORES AND UNPIGMENTED CELLS PER UNIT AREA TO THE DEVELOPMENT OF THE LAYERS OF THE SKIN IN LARVAL AND ADULT *Ambystoma maculatum*

Length (Mm.)	Description	Subdermis			Dermis				Epidermis	Total	
		Unpigmented Cells		Melanophores	Unpigmented Cells		Melanophores	Thickness (μ)	Melanophores	Unpigmented Cells	Melanophores
		Number	Mitotic Index		Number	Mitotic Index					
14	Larva	81	6.4	10	0	0	0	3	11	81	21
20	Larva	117	2.5	15	0	0	0	3	8	117	23
24	Larva	125	0.6	11	0	0	0	7	7	125	18
27	Larva	121	5.0	9	1	0	0	10	4	121	13
32	Larva	158	.....	15	2	.....	0	13	8	160	23
36	Larva	165	5.7	19	6	12.9	2	18	9	163	30
39	Larva	116	3.3	6	43	7.5	5	16	6	159	16
42	Larva	107	.....	2	120	.....	16	25	9	227	27
45	Larva	122	.....	0	147	.....	18	46	6	269	24
46	Larva	95	0.2	0	168	0.4	19	66	6	263	25
48	Metam. I	94	.....	0	171	.....	18	69	11	265	29
48	Metam. II	110	.....	0	216	.....	35	60	11	326	46
47	Adult I	76	.....	1	162	.....	49	68	7	238	57
48	Adult II	58	.....	0	148	.....	28	75	11	206	39
60	Adult III	69	.....	2	182	.....	61	91	5	251	68

crease in number of subdermal cells. This is accompanied by an increase in the thickness of the dermis and a looser arrangement of the fibers. As this degree of development is reached, a capillary net is established in the dermis for the first time. Skin glands begin to appear, first, as small buds that are downgrowths of the basal layer of the epidermis. The anlagen increase in size rapidly, and, as they push down into the dermis, they appear to push the collagenous fiber layer down before them. This results in a region of loose connective tissue which will soon make up the greatest part of the dermis and in which the cells accumulate. The increase in number of dermal unpigmented cells during this time may, in large part, be accounted for by the high mitotic index of these cells. Although it is probable that the greatest part of any further increase in number of dermal unpigmented cells is the result of mitoses, throughout the larval period and at times even in the adult one may see subdermal unpigmented cells partially imbedded in the fibrous inner layer of the dermis. The distorted shape of the nuclei of many of these cells strongly suggests that these cells are migrating from a subdermal position into the dermis.

Although the larvae become progressively darker as they approach metamorphosis, there is no corresponding increase in the number of melanophores per unit area. However, the darkening color may be correlated with the change in position of the melanophores, which now occur principally in the dermis instead of in the subdermis. This more superficial position under the epidermis may make them more visible, so that they are more conspicuous externally. There has been, however, a large increase in the number of unpigmented cells, which appear to be concerned in the development of the dermis. The subdermal region, at this time constituting the loose connective-tissue layer under the dermis, contains only a small number of expanded unpigmented cells and an occasional melanophore.

In the advanced larva, which is soon to metamorphose, the dermis is relatively thick and loose and consists of three layers. Immediately below the epidermis there is a thin dense layer of connective-tissue fibers, that contains no cells and is interrupted only by the ducts of the skin glands. Below this, a layer of loose connective tissue forms the major part of the dermis and contains the bodies of the skin glands, now fairly well differentiated, and the dermal cells. The loose network of fibers which fills the area between the glands contains melanophores and unpigmented cells, as well as numerous capillaries. The deepest layer of the dermis consists of densely arranged connective-tissue fibers, in which are imbedded a few unpigmented cells but no melanophores and which is penetrated by the capillaries. This fibrous layer sharply delimits the dermis from the underlying regions.

The criteria of beginning metamorphosis in the living animal are arbitrary ones, but for this purpose they will be considered to be the first indication of the reduction of the gills and the dorsal fin. These changes are noted in *A. tigrinum* at about 100 mm., while in *A. maculatum* they appear at about 50 mm. There is, however, no striking change in the histological structure of the skin corresponding to the beginning of external metamorphic changes. The increase in the number of dermal melanophores which occurs during metamorphosis is begun in the advanced larva. The melanophores, dispersed throughout the loose layer of the dermis, are large cells with greatly expanded processes. In *A.*

tigrinum a gradual increase in number of dermal unpigmented cells occurs during metamorphosis, but in *A. maculatum* any significant increase in number ceases prior to the beginning of metamorphosis. The dermis becomes thicker, chiefly through further increase in the intermediate layer, although the inner layer of densely packed fibers becomes thicker also. The subdermal region is not changed from the premetamorphic condition. The skin glands increase in size during metamorphosis and complete their differentiation. Grossly, the skin acquires a smoother appearance, probably as a result of the disappearance of the Leydig cells. In *A. tigrinum* melanophores are evenly distributed over the back and flanks, so that the dorsal surface becomes uniformly black, in contrast to the yellowish-green of the young larva. In *A. maculatum*, however, small, indistinct yellow spots appear on the black ground color of the dorsal surface. These become clearer and less numerous, so that they are fairly large and distinct when metamorphosis is completed.

There is a shift in the arrangement of melanophores in the dermis at the completion of metamorphosis. In the adult that has just completed metamorphosis, melanophores are arranged in the outer part of the loose fibrous region to form a fairly complete network beneath the thin outer layer of the dermis, and only a few processes are found deeper in the dermis. The layer of melanophores is continued around the glands so that they are enveloped by a network of melanophores. In adults of about 2 months, the oldest animals in these series, the number of dermal melanophores reaches the highest values observed here. The skin glands are large and occupy the major part of the dermis, and their cells contain a large amount of elaborated secretion. The dermis is very thick (325 μ in *A. tigrinum;* 90 μ in *A. maculatum*) when compared to the young larva, whose dermis is only about 4 μ thick. Unpigmented cells are rarely found immediately below the dermis. Subdermal melanophores have become more numerous, perhaps significantly so, but their position appears to correspond to the presence of blood vessels and may have no relation to the pigmentation of the skin. These features are brought out in *D*, Plate I, which shows the structure of the skin of the adult *A. tigrinum*. The adult coloration of this species is a black ground color over the back, with irregularly distributed yellow or cream spots. The ventral surface is a dark gray and contains a large number of cream spots. This is the definitive pattern of this species and will persist throughout the life of the animal. The adult coloration of *A. maculatum* is an intense black on the dorsal surface and flanks and a dark gray on the ventral side. The yellow spots are now clearly outlined and form two rows along the entire length of the back.

Tables 1 and 2, previously referred to, give in detail the exact number of cells per unit area for each animal in the series. The totals of dermal and subdermal unpigmented cells are included to show the change in number of this cell type during development. In summing these cells, it is assumed, of course, that subdermal and dermal unpigmented cells are related, a conclusion suggested by the correspondence in time between the decrease in number of subdermal unpigmented cells and the first appearance and subsequent increase in dermal cells of the same type. Further evidence in support of this hypothesis is the frequent appearance at this time of subdermal cells with most of the cell body in the dermis. That they are migrating from the subdermis into the dermis is the only explanation which presents itself; it is

borne out by all the observed facts and contradicted by none. The justification for summing subdermal, dermal, and epidermal melanophores, also included in the tables, follows the same reasoning as that used for the unpigmented cells. Melanophores which appear to be migrating into the dermis are not infrequently seen during the time when unpigmented cells appear to be migrating most actively. Dermal or subdermal melanophores with processes extending into the epidermis are seen, but less frequently.

In *A. tigrinum*, the total number of unpigmented cells slowly increases during larval development and more rapidly as metamorphosis approaches (Fig. 1). The total number of melanophores shows no increase per unit area until after metamorphosis has begun. During advanced metamorphosis there is a rapid increase which corresponds to the time at which the animal rapidly darkens. In *A. maculatum* unpigmented cells increase in number during the larval period, but during metamorphosis the number decreases. The total number of melanophores shows no significant change until metamorphosis begins, but after this the increase is fairly rapid, so that a comparatively high count is obtained in the adult. It is possible that part of the decrease in number of unpigmented cells which appears to occur at this time may be accounted for by their transformation into melanophores. Although there is no direct evidence here to support this suggestion, it has been shown that donor melanophores arise in grafts which previously have been pigment free (DuShane, 1943) and therefore must have differentiated from unpigmented cells. Since mitoses in melanophores are uncommon, the rapid increase in these cells that occurs at the end of metamorphosis and in the young adult can be explained most satisfactorily as resulting from the transformation of unpigmented cells. One may point out, it is true, that a corresponding decrease in unpigmented cells does not occur in *A. tigrinum*. In *A. tigrinum*, however, the number of unpigmented cells reaches a much higher value, while the increase in number of melanophores is slower and they do not become as numerous as in *A. maculatum*. For these reasons the increase in number of melanophores may not be so obviously reflected in a decrease in number of unpigmented cells as in *A. maculatum*.

The relation between dermal and subdermal cells is indicated in Figures 1 and 2, in which are graphed three-point running averages of some of the data presented in Tables 1 and 2. Mitotic indexes of subdermal and dermal unpigmented cells are also plotted. The increase in dermal thickness that occurs during the developmental period under consideration here are shown in *A* and *B*, Figure 4.

T. torosus

The development of the melanophore pattern in this species differs from that found in *Ambystoma*. The first melanophores develop along the back to form a band on either side of the dorsal fin. Melanophores first differentiate in the subdermis and appear in the embryo at about stage 35. Their processes may be found extending along the undersurface of the dermis and at intervals give off fine branches into the epidermis. The development of chromatophores is slightly more advanced in the anterior region. At stage 37, more of the melanophores in the anterior trunk are fairly well pigmented, while more posteriorly they contain fewer pigment granules. This leaves little doubt that melanophores are pigmented *in situ* and do not, as Berweger

(1926) suggested, acquire their pigment only in the anterior part of the body. Epidermal melanophores are found only after those in the dermis have become fairly numerous. By the time the larvae are free-swimming, the subdermal melanophores are fully pigmented and are very massive, with heavy processes which are packed with large melanin granules.

A peculiar relationship is seen between the subdermal melanophores that form the dorsal bands and the melanophores that are found around the spinal cord.

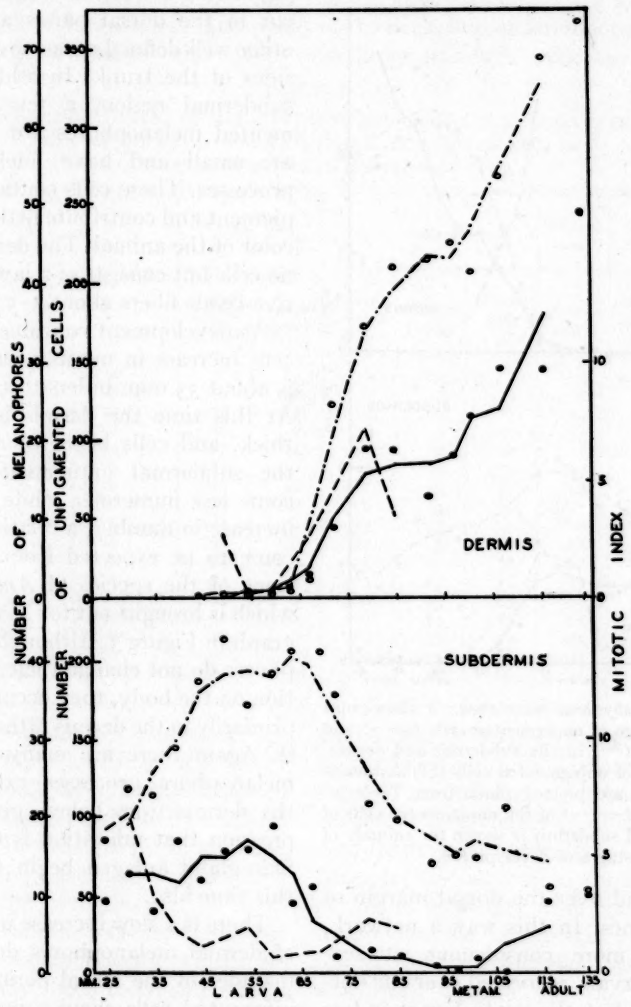

FIG. 1.—*Ambystoma tigrinum.* A three-point running average of unpigmented cells (•-•-•) and melanophores (—) in the subdermis and dermis. Actual values of unpigmented cells (○) and melanophores (●) are plotted (data from Table 1). Mitotic index (– – – – –) of the unpigmented cells of the subdermis and dermis is shown for animals of corresponding stages of development.

The subdermal melanophores occur at the dorsal boundary of the myotomes. These bands are connected to each other and to the melanophores surrounding the spinal cord by other melanophores,

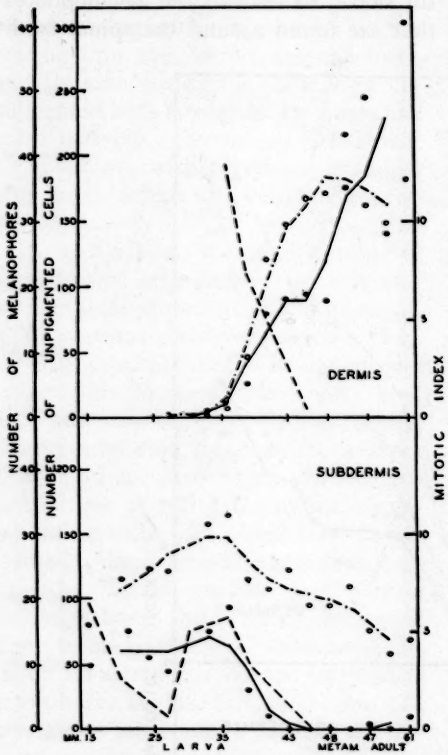

FIG. 2.—*Ambystoma maculatum*. A three-point running average of unpigmented cells (•----•) and melanophores (—) in the subdermis and dermis. Actual values of unpigmented cells (○) and melanophores (●) are plotted (data from Table 2). Mitotic index (– – – –) of the unpigmented cells of the dermis and subdermis is shown for animals of corresponding stages of development.

which extend over the dorsal margin of the myotomes. In this way a network, sometimes more conspicuous at segmental intervals, is formed over the dorsal surface; but the central part is less apparent in the living animal because of its deeper position in the connective tissue (see *A*, Pl. II).

This species shows the same general pattern in the formation of the dermis as that found in the species of *Ambystoma* which have been described, but there is some modification in the development of the pigmentary pattern. During the early part of the larval period the heavily pigmented subdermal melanophores occur in the dorsal bands and in a few other well-defined areas on the head and sides of the trunk. In addition, in the subdermal region, a few faintly pigmented melanophores are found which are small and have finely branching processes. These cells contain very little pigment and contribute little to the gross color of the animal. The dermis contains no cells but consists of a layer of connective-tissue fibers about 3–5 μ thick.

As development continues, subdermal cells increase in number until the larva is about 33 mm. in length (see Table 3). At this time the dermis is about 17 μ thick, and cells begin to appear. Soon the subdermal unpigmented cells become less numerous, while dermal cells increase in number, a relation which has come to be expected from the descriptions of the species of *Ambystoma* and which is brought out for *T. torosus* in the graph in Figure 3. Although the melanophores do not change their relative position on the body, they occur at this time primarily in the dermis rather than below it. Again there are many instances of melanophore processes extending into the dermis from below, giving the impression that migration is taking place. Skin-gland anlagen begin to appear at this time also.

There is a slow increase in the number of dermal melanophores during the remainder of the larval period, while unpigmented cells show a more rapid increase. In the advanced larva, shortly before metamorphosis, subdermal unpigmented cells are less numerous, and

melanophores are extremely rare. The dermis is now more than 70 μ thick, and the mid-dermal layer contains loosely arranged collagenous fibers, in which numerous unpigmented cells occur. The dorsal bands have become wider, but melanophores are confined, for the most part, to these lines. Skin glands are large and fairly well differentiated, and the dermis extends downward where they occur. Many capillaries penetrate the inner dermal layer and form a network in the loose middle layer. In the advanced larva the epidermis begins to darken and to take on a brownish hue, in contrast to the transparent greenish-yellow which characterizes the young larva. The darkening results from the increase in number of epidermal melanophores, which, although they have been increasing slowly during the larval period, become more numerous at this time. This is accompanied by an increase in the amount of melanin in the epidermal cells.

With the onset of metamorphosis, unpigmented cells as well as melanophores occur in the outer portion of the mid-dermal layer where their processes form a network below the epidermis. In many cases dermal melanophore processes extend into the epidermis. The dorsal bands have expanded over a larger area of the back, and melanophores also occur in large numbers over the head and flanks. The dermis has greatly increased in thickness, but in a more advanced stage of metamorphosis it becomes condensed. These changes in dermal thickness are shown graphically in *C*, Figure 4. After it has become so condensed, the dermis is no longer wide enough to accommodate the larger skin glands. As a result, the inner dermal layer extends downward around the body of each gland, and the epidermis protrudes outward. In addition, local thickenings of the epidermis occur where the epidermis extends over the glands. It is the aggre-

TABLE 3

RELATION OF THE NUMBER OF MELANOPHORES AND UNPIGMENTED CELLS PER UNIT AREA TO THE DEVELOPMENT OF THE LAYERS OF THE SKIN IN LARVAL AND ADULT *Triturus torosus*

Length (Mm.)	Description	Subdermis		Dermis			Epidermis	Total	
		Unpigmented Cells	Melanophores	Unpigmented Cells	Melanophores	Thickness (μ)	Melanophores	Unpigmented Cells	Melanophores
15	Larva	57	10	0	0	3	0	57	10
18	Larva	74	10	0	0	5	2	74	12
21	Larva	103	10	0	0	7	4	103	14
24	Larva	88	9	0	0	10	3	88	12
27	Larva	111	14	0	0	10	2	111	16
30	Larva	117	22	0	0	12	7	117	29
33	Larva	126	26	1	0	17	6	127	32
36	Larva	134	18	6	1	20	11	140	30
39	Larva	121	16	13	2	20	9	134	27
42	Larva	101	8	27	9	17	18	128	35
45	Larva	78	0	215	11	18	17	293	28
46	Larva	43	0	221	13	76	16	264	29
44	Metam. I	49	0	280	13	86	29	329	42
49	Metam. II	52	0	416	33	111	13	468	46
45	Metam. III	46	0	310	33	50	12	356	45
47	Adult I	32	0	382	21	51	24	414	45
45	Adult II	49	0	309	24	69	44	358	68

gate of these two factors which results in the irregular tubercles which are characteristic of adult *T. torosus* epidermis.

Grossly, the young adult appears uniformly brown, and there is no longer any indication of the dorsal bands. Histologically, the subdermal region is unchanged from that found in the advanced larva. The dermis, however, has become slightly thicker than it was during metamorphosis, approximately 70 μ. The number of dermal unpigmented cells shows what may be a significant decrease in number, when compared with the number found during metamorphosis. Dermal melanophores are significantly decreased at this time and are extremely contracted, and the short processes are densely packed with melanin granules. Only rarely can a process be seen extending into the epidermis. These melanophores occur everywhere except on the ventral surface but, because of their extreme contraction, contribute little to the gross coloration of the animal. Some cells are found in the dermis, however, which contain only a few melanin granules. These cells may be developing melanophores, since the nucleus appears normal and the cell as a whole does not show any degenerative changes. The granules are small, more brownish in color, and resemble those of a developing melanophore in the embryo. Epidermal melanophores have again become very abundant and occur as highly branched cells with far-reaching processes. It may be possible to correlate the increase in epidermal melanophores with the decrease in number of dermal melanophores observed at this time. Because of their great expansion, epidermal melanophores may be easily confused with the ordinary epidermal cells, which also contain a large amount of melanin granules. The melanin occurs throughout the cytoplasm of these cells, but it is particularly abundant on the outer side of the nucleus. These characteristics of the adult *T. torosus* skin are shown in *B*, Plate II.

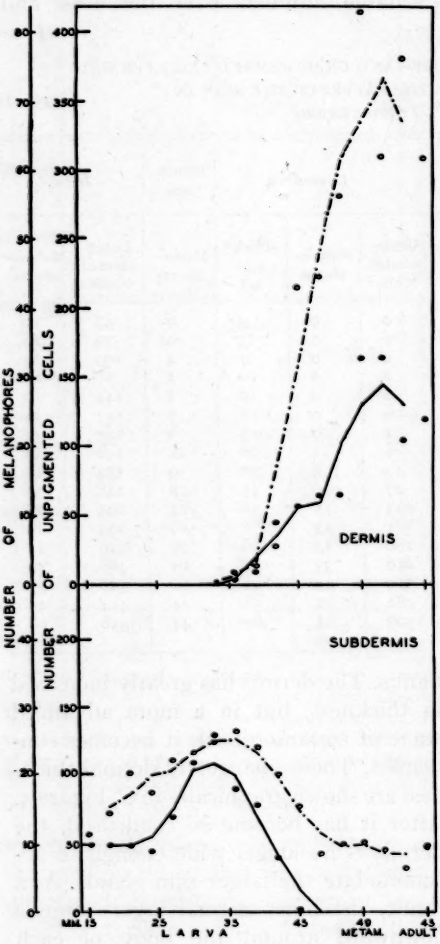

FIG. 3.—*Triturus torosus*. A three-point running average of unpigmented cells (•--•--•) and melanophores (—) in the subdermis and dermis. Actual values of unpigmented cells (○) and melanophores (●) are plotted (data from Table 3).

Table 3 gives the total number of unpigmented cells per unit area—subdermal and dermal—for each animal of the series. There is a slow increase during most of the larval period and a more

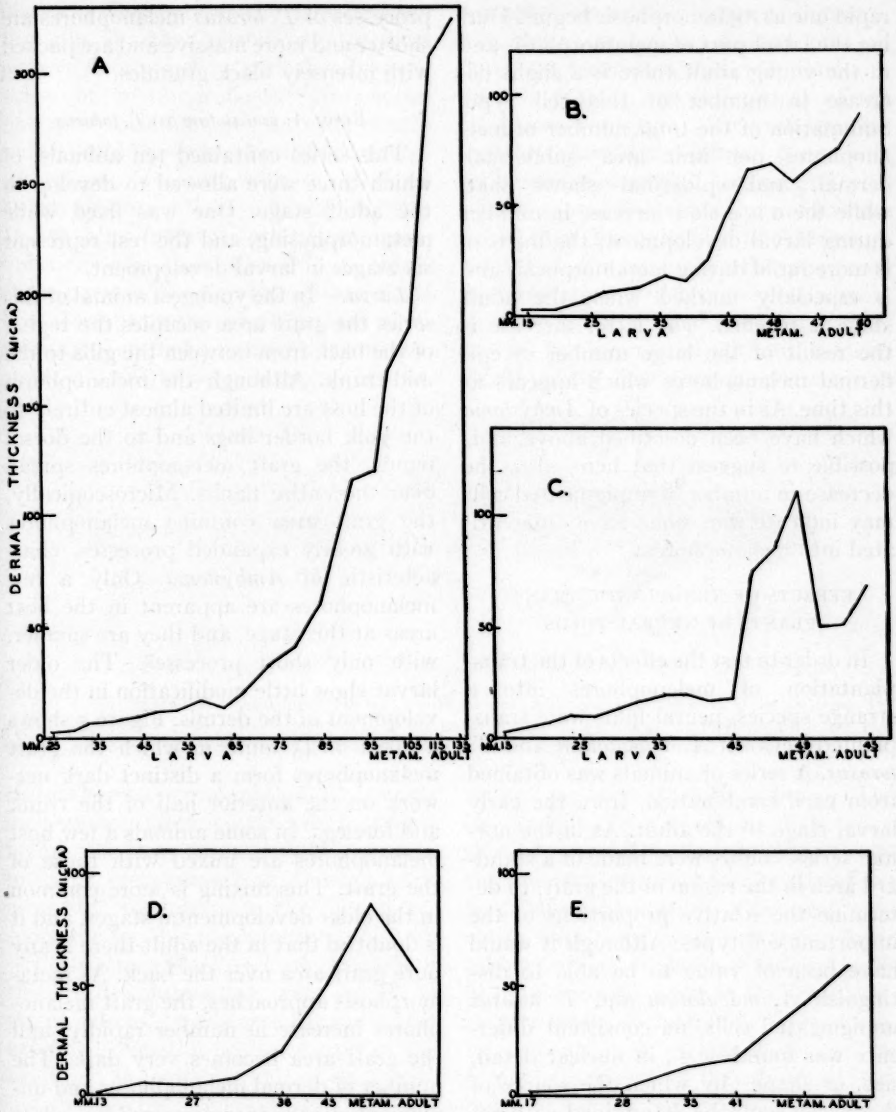

FIG. 4.—The increase in the thickness of the dermis during development in five series. *A*, *Ambystoma tigrinum; B*, *Ambystoma maculatum; C*, *Triturus torosus*, *D*, *T. torosus* with grafted neural crest from *A. maculatum; E*, *A. maculatum* with grafted neural crest from *T. torosus*.

rapid one as metamorphosis begins. During the latter part of metamorphosis and in the young adult there is a slight decrease in number of this cell type. Summation of the total number of melanophores per unit area—subdermal, dermal, and epidermal—shows that, while there is a slow increase in number during larval development, the increase is more rapid during metamorphosis and is especially marked when the adult stage is reached. The latter increase is the result of the large number of epidermal melanophores which appears at this time. As in the species of *Ambystoma* which have been described above, it is possible to suggest that here, also, the decrease in number of unpigmented cells may indicate that some have differentiated into melanophores.

EFFECTS OF XENOPLASTIC TRANSPLANTS OF NEURAL FOLDS

In order to test the effects of the transplantation of melanophores into a strange species, neural folds were transplanted between *A. maculatum* and *T. torosus*. A series of animals was obtained from each combination, from the early larval stage to the adult. As in the normal series, counts were made of a standard area in the region of the graft, to determine the relative proportions of the important cell types. Although it would have been of value to be able to distinguish *A. maculatum* and *T. torosus* unpigmented cells, no consistent difference was found—e.g., in nuclear detail, size, or shape—by which the source of these cells could be determined with accuracy. However, the melanophores of the two species are sufficiently different to permit them to be readily distinguished. Those of *A. maculatum* have more finely branched processes, the melanin granules are not quite so black, and they appear to be smaller. The processes of *T. torosus* melanophores are shorter and more massive and are packed with intensely black granules.

FROM *A. maculatum* TO *T. torosus*

This series contained ten animals, of which three were allowed to develop to the adult stage. One was fixed while metamorphosing, and the rest represent six stages in larval development.

Larva.—In the youngest animal of this series the graft area occupies the region of the back from between the gills to the mid-trunk. Although the melanophores of the host are limited almost entirely to the yolk border lines and to the dorsal bands, the graft melanophores spread over the entire flanks. Microscopically, the graft area contains melanophores with greatly expanded processes, characteristic of *Ambystoma*. Only a few melanophores are apparent in the host areas at this stage, and they are smaller, with only short processes. The older larvae show little modification in the development of the dermis. Figure 5 shows a larva of 45 mm., in which the graft melanophores form a distinct dark network on the anterior half of the trunk and forelegs. In some animals a few host melanophores are mixed with those of the graft. This mixing is more common in the older developmental stages, and it is doubtful that in the adult there is any pure graft area over the back. As metamorphosis approaches, the graft melanophores increase in number rapidly, and the graft area becomes very dark. The number of dermal melanophores and unpigmented cells increases rapidly, while the number of subdermal cells decreases slowly. The cells of the dermis attain their maximum number during metamorphosis and are slightly decreased when the adult stage is reached. In the metamorphosing animal of this series the graft area is almost black, with a fairly

distinct yellow spot on the back between the gills. Farther posteriorly the host pattern does not show such an advanced stage of metamorphosis. The dorsal bands of melanophores are still clearly visible, and the general color is a greenish brown, in contrast to the uniform brown pattern of the adult *T. torosus*. It has been found in other graft combinations also that the adult *A. maculatum* pattern develops before that of the host. It appears that the threshold of reactivity of grafted cells might be lowered, since, as will be seen later, in the reciprocal graft combination *T. torosus* melanophores assume the adult pattern before the transformation is complete in the *A. maculatum* host. Further work is needed to determine the factors responsible for this phenomenon.

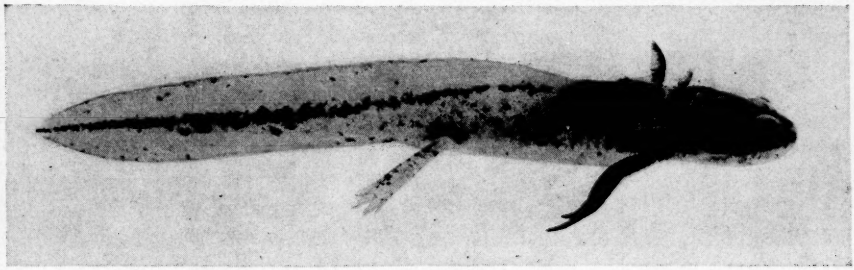

FIG. 5.—Advanced *T. torosus* larva (45 mm.) with neural crests transplanted from *A. maculatum*. The graft melanophores form a black network over the anterior trunk region and the forelegs, while the host melanophores in the posterior trunk and tail form the dorsal bands characteristic of *T. torosus*.

Postmetamorphosis.—Three animals of this graft combination were raised to young adults. In all cases the graft area extended over the posterior part of the head and the anterior half of the trunk, in two cases the forelegs showed the characteristic black of the donor, and in one case the graft melanophores extended to the mid-ventral line, so that a black strip was formed across the ventral surface between the forelegs. This is in sharp contrast to the normal host pattern, in which melanophores never occur on the ventral surface. This animal, shown in Figure 6, had the most clearly defined area of donor pigmentation and was therefore used in making the cell counts of the adult (see Table 4, *a*). The graft area invariably showed the normal donor pattern—an intense black background with yellow spots. The spots, however, were not always clearly defined and, although they tended to be smaller, they appeared in the locations which correspond closely to those in the normal donor, as nearly as can be determined without detailed study. In the host area the pattern was that of the normal adult *T. torosus*. The tail and posterior trunk in these cases showed no graft melanophores but only the uniform brown, characteristic of the host. Microscopic study of the graft area shows that the dermis contains a large number of melanophores, which are highly branched and occur in the loose connective tissue of the middle layer. Rarely, a contracted melanophore can be seen, characteristic of the adult host; but much less epidermal pigment is present than is found in the normal *T. torosus*. In the mid-dorsal region a small area of graft epidermal cells (from *A. maculatum*) contains less melanin than the surrounding host cells (see *D*, Pl. II). The The skin glands which have formed from the graft epidermis are characteristic of

the donor and show consistent differences from those of the host. The dermis in the area of host pigmentation contains only a few melanophores which are greatly contracted, although a few fine processes extend into the epidermis. More melanin is present in the epidermal cells than was found in the area of graft melanophores.

Table 4, *a*, gives the actual values obtained from cell counts in the animals in this series. That the relation between important cell types is unchanged from that found in the normal series can be seen in Figure 7. From this graph it is apparent that the strange host environment has essentially the same influence upon the graft cells as has their normal environment. Dermal thickness, included in Table 4, is graphed in *D*, Figure 4. It can be readily seen that dermal development, if increase in thickness is used as a criterion, closely follows that found in the normal *T. torosus*. It is apparent, therefore, that the presence of foreign (graft) melanophores, as well as unpigmented cells within the dermis, does not alter its normal pattern of development.

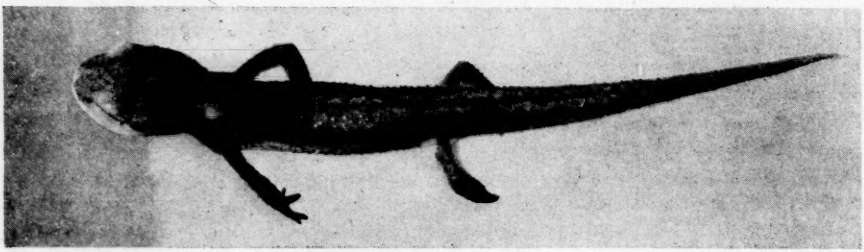

FIG. 6.—Adult *T. torosus* with neural crests transplanted from *A. maculatum*. Graft melanophores are evenly distributed over the anterior trunk region and forelegs. Note the small spot, characteristic of *A. maculatum*, on the left shoulder.

FROM *T. torosus* TO *A. maculatum*

Of the six animals with this graft combination, one was carried to the young adult stage, one was fixed while undergoing metamorphosis, and the remaining four were fixed at representative stages in larval development. In only one or two instances was it possible to obtain a graft area of *T. torosus* cells which was not invaded by melanophores of the host. Apparently, *A. maculatum* cells have a greater capacity to migrate, and they invade areas of *T. torosus* cells very readily.

Larva.—The youngest animal of the series shows a clear graft area over the entire trunk. Melanophores on the dorsal surface form fairly distinct dorsal bands, and almost no epidermal melanophores are present in this area. Histologically, graft melanophores are densely pigmented and somewhat contracted, in contrast to the larger, more expanded host melanophores. The next animal of the series has an indistinct graft area. The trunk contains a few dermal melanophores, which are darker and more contracted than the surrounding host melanophores. There is no essential change in histological detail when compared to the normal, and there are probably very few graft cells in the area. In the more advanced larvae of the series the graft area is somewhat clearer, although some host chromatophores are present. Grossly, the dorsal bands are

somewhat indistinct, and the area around them is lighter than the corresponding area in the host (fewer and more contracted melanophores) but darker than is found in the donor of the corresponding stage of development. The dermis develops normally, but the larval pigmentary pattern of the donor does not develop to the extent seen in the reciprocal graft combination.

In the animal which was fixed during metamorphosis, the graft area was clearly defined and extended over the entire trunk. At fixation, this region was a light gray, except for two or three areas over the back, which were darker and evidently contained host chromatophores. Here, again, the graft attains the adult pattern before this stage of development is reached in the host. A satisfactory explanation of this phenomenon must await further investigation. Nevertheless, this gray donor pattern is not quite characteristic of *T. torosus*. Microscopic study shows that the dermis contains melanophores which are almost completely contracted. Almost no pigment is present in the epidermal cells, and epidermal melanophores are extremely rare. It is the absence of epidermal pigment, as well as the presence of only contracted melanophores in the dermis, which is reponsible for the gray appearance of the graft area.

Postmetamorphosis.—The only adult in this series shows a small but fairly distinct graft area on the back between the forelegs, as well as on the forelegs

TABLE 4

GRAFT SERIES: RELATION OF NUMBER OF MELANOPHORES AND UNPIGMENTED CELLS PER UNIT AREA TO THE DEVELOPMENT OF THE LAYERS OF THE SKIN IN LARVAL AND ADULT ANIMALS

Length (Mm.)	Description	Subdermis		Dermis			Epidermis	Total	
		Unpigmented Cells	Melanophores	Unpigmented Cells	Melanophores	Thickness (μ)	Melanophores	Unpigmented Cells	Melanophores
	a) *T. torosus* with *A. maculatum* neural crest								
13	Larva	77	13	0	0	2	1	77	14
21	Larva	98	13	0	0	6	4	98	17
27	Larva	135	12	0	0	5	2	135	14
31	Larva	127	12	0	0	8	5	127	17
36	Larva	83	14	21	8	20	3	104	25
45	Larva	73	6	141	38	50	20	214	64
51	Metam.	92	12	458	86	86	10	550	108
49	Adult	18	4	280	77	56	19	298	100
	b) *A. maculatum* with *T. torosus* neural crest								
17	Larva	80	8	0	0	2	1	80	9
28	Larva	140	9	0	0	3	5	140	14
33	Larva	95	7	33	3	13	2	128	12
41	Larva.	97	4	44	19	16	6	141	29
49	Metam.	73	4	154	50	41	1	227	55
58	Adult	83	4	252	44	59	4	335	52

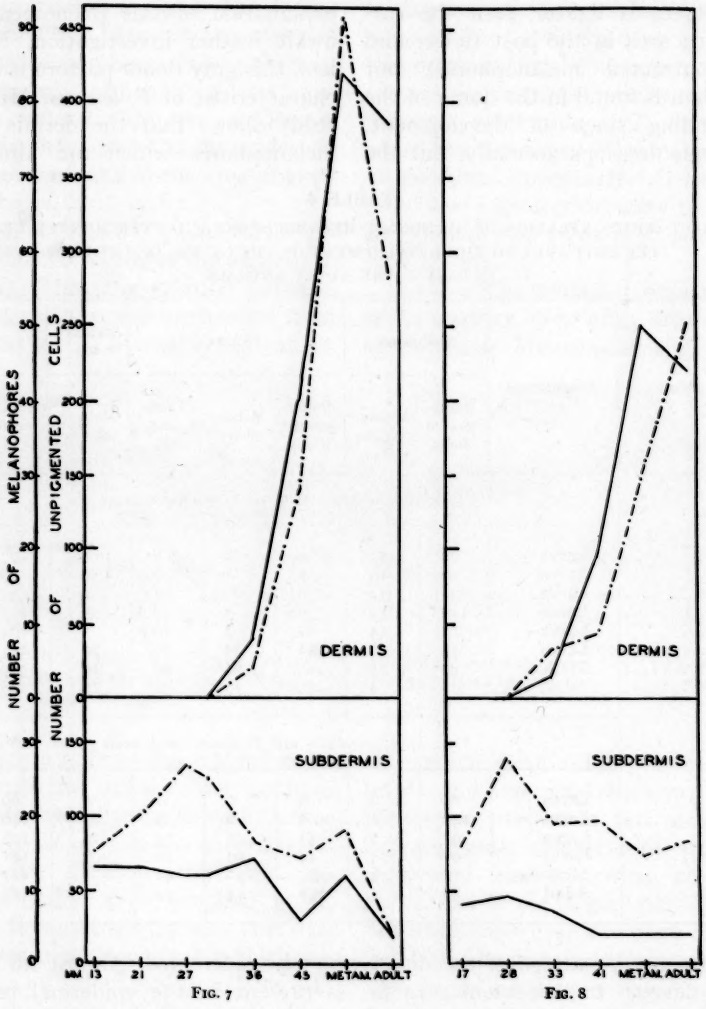

FIG. 7 FIG. 8

FIG. 7.—*Triturus torosus* with neural crests grafted from *A. maculatum*. Curves show the number of unpigmented cells (•-•-) and melanophores (—) in the subdermis and dermis for each animal in the series.

FIG. 8.—*Ambystoma maculatum* with neural crests grafted from *T. torosus*. Curves show the number of unpigmented cells (•-•-) and melanophores (—) in the subdermis and dermis for each animal in the series.

themselves. This area on the body appears light gray, and the legs are even lighter. The surrounding host skin is quite black, and distinct yellow spots are evident. Grossly, the grafted melanophores in the dermis appear as dots, while histologically they are seen to be extremely contracted. Expanded melanophores also occur in this area and are probably of host origin. As in the preceding animal, the epidermis in the area of the graft melanophores is almost completely devoid of melanophores, as well as melanin, in the epidermal cells. However, a few donor epidermal cells which are present in the mid-dorsal region contain large amounts of melanin granules (see *C*, Pl. II). The forelegs are even more free of host melanophores and epidermal pigment and therefore appear even lighter in color. Table 4, *b*, lists the values obtained in cell counts in the animals of this series. Although the number of animals is small, Figure 8 shows that the relation between dermal and subdermal unpigmented cells and melanophores is essentially the same in this graft combination as has been found in the normal host, *A. maculatum*. The graft cells appear to have no influence upon the development of the dermis, at least not when thickness is used as a criterion.

Epidermal Pigmentation

The origin of epidermal melanophores, as well as the source of pigment in the epidermal cells, has been the subject of much controversy. For this reason special attention has been given to this point in the material available in the present study. Many instances have been found of the migration of melanophores into the epidermis from the deeper layers of the skin, and there is a good correlation between the presence of expanded melanophores and the occurrence of melanin in epidermal cells.

As has been mentioned briefly above, the epidermis of the adult *T. torosus* contains large amounts of melanin, both in epidermal cells and in melanophores. This condition is attained by a developmental process which extends throughout the larval period (see *A*, Fig. 9). In the embryo, melanophores first occur only in the subdermal region, at a time when only egg pigment is present in the epidermal cells. Although this pigment may be confused with the pigment occurring in melanophores, the two may be distinguished by the fact that the melanin granules found in chromatophores do not occur in such a finely divided state as does egg pigment. In the larva and adult the melanin occurring in epidermal cells is more likely to be accumulated in a particular part of the cell than to be evenly distributed throughout the cytoplasm. The major part of this melanin is usually aggregated to form a cap over the outer side of the nucleus, while the remaining granules are distributed more evenly and sparsely in the rest of the cytoplasm. Soon after subdermal melanophores begin to appear in the *T. torosus* embryo, their processes are seen extending into the epidermis. This is followed by an increase in epidermal pigment, especially in the regions around melanophore processes. However, only a few epidermal melanophores and little epidermal melanin is present during the early larval period, and it is not until the larvae reach a length of 30–35 mm. that epidermal melanophores become much more numerous. There is little epidermal pigment in the vicinity of contracted melanophores; but, when the melanophores are expanded, the epidermal cells contain more pigment. In the older larvae there is a further increase in epidermal melanophores and in the melanin found in epidermal cells. These

melanophores are extremely expanded, and their fine, highly branched processes extend for long distances between the epidermal cells. At their ends many of the processes penetrate the epidermal cells and pass over the outer side of the nucleus to form a cap. In many instances it is possible to see a fine line of granules at the end of the melanophore process.

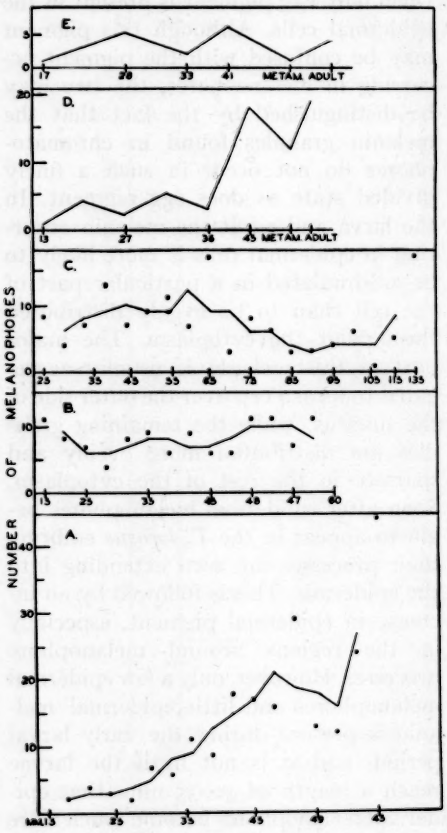

FIG. 9.—Curves show the variation in number of epidermal melanophores during development. *A*, *T. torosus; B*, *A. maculatum*, *C*, *A. tigrinum; D*, *T. torosus* with grafted *A. maculatum* neural crests; *E*, *A. maculatum* with *T. torosus* neural crests. In *A*, *B*, and *C*, the graph represents a three-point running average of actual values, while the dots represent observed values of each animal in the series (data from Tables 1, 2, and 3). In *D* and *E* the graph shows actual values (data from Table 4).

This condition is seen perhaps best in the advanced *T. torosus* larva (see *E*, Pl. II) at the time when epidermal pigmentation is increasing most rapidly. At the beginning of metamorphosis the epidermis is heavily pigmented, and the relation of numerous epidermal melanophores, and much epidermal pigment which is present at this time persists throughout adult life. As for the source of epidermal melanophores, one may find evidence of migration of melanophores into the epidermis from deeper positions during the entire larval period, but it is most commonly seen at the time when the number of epidermal melanophores is increasing most rapidly.

In the other species studied here, there is no regular development of epidermal pigmentation as in *T. torosus*. In *Ambystoma* some epidermal melanophores are always present, but there is considerable variation in number in the larva as well as in the adult (see *B* and *C*, Fig. 9). When the epidermal melanophores are contracted, there is usually a complete absence of epidermal pigment, while, when they are highly expanded and many processes enter the epidermal cells, there is a large quantity of epidermal pigment. In all cases epidermal pigment can be found only in the part of the epidermis in which melanophores are present. It has also been noted that epidermal melanin is more abundant where many melanophore processes enter the epidermal cells. Tangential sections of *A. tigrinum* and *A. maculatum* epidermis show finely branched melanophores with processes penetrating many of the surrounding epidermal cells. These processes appear to break up within the epidermal cells and to form a cap over the outer side of the nucleus. (In sections stained with thionine, it is seen that lipophores as well as melanophores send finely branched processes for some dis-

tance between the cells of the epidermis and commonly enter these cells, as do the processes of the melanophores.)

As further evidence of the relation between epidermal pigment and melanophores, a study was made of other species in which a special attempt was made to compare dorsal and ventral epidermis. In *T. pyrrhogaster* the ventral skin usually contains no epidermal melanophores, and there is no pigment in the epidermal cells. However, one small epidermal melanophore was found, and the epidermal cells for a short distance around it contained a small amount of melanin granules. *Triturus granulosus* also shows no epidermal melanophores on the ventral surface and no melanin granules in the epidermal cells. On the flanks an occasional melanophore may be found, as well as some epidermal pigment, while farther dorsally more epidermal melanophores are present and there is a large amount of epidermal melanin. Again, in *T. rivularis*, there is a high degree of correlation between the presence of epidermal pigment cells and epidermal pigment.

In the animals with grafted neural crests there is additional evidence of the relation between epidermal cell pigment and melanophores. The area of graft melanophores in adult *T. torosus* with neural crests from *A. maculatum* contains, in most cases, a few host melanophores, but much less than is normally found. It is relatively easy to differentiate host and donor dermal melanophores, since those from *T. torosus* are always extremely contracted in the adult, while those from *A. maculatum* are expanded. However, it was possible to obtain only graft chromatophores in the forelegs of one adult *T. torosus* host, and in this case there was no evidence of the migration of grafted *A. maculatum* dermal melanophores into the epidermis, and neither epidermal melanophores nor epidermal pigment was found there. Where a small area of graft epidermis is present (in the mid-dorsal cervical region), these cells contain the normal amount of melanin, and melanophores are present. The normal pigmentation in the graft epidermis indicates that this phenomenon is not the result of some effect of the strange environment of the host upon the graft melanophores. In *D*, Plate II, the sharp line of demarcation between the epidermis of graft and of host origin is shown. The donor epidermal cells contain a small amount of epidermal pigment, characteristic of normal *A. maculatum*, while the melanin in the host epidermis is strikingly reduced as compared to the normal host, *T. torosus*. Counts of epidermal melanophores in this series, graphed in *D*, Figure 9, show that there is an increase in number during development similar to that found in the normal *T. torosus* but that in the graft animals the increase is not so great. The small number of host melanophores, which are present in the region dominated by graft melanophores, is probably the source of the slight increase in epidermal pigment which is observed.

The reciprocal graft combination illustrates equally well the inability of graft melanophores to pigment the epidermis of the host. Although the melanophores of *T. torosus* are normally able to produce the heavily pigmented epidermis, only a few epidermal melanophores and a small amount of epidermal pigment are present where these melanophores are covered by host (*A. maculatum*) epidermis. In *C*, Plate II, the same relation as that noted above is shown. The small area of graft epidermis is heavily pigmented, while the adjoining host epidermis is essentially pigment-free. The counts of epidermal melanophores recorded for this series are

graphed in *E*, Figure 9, and show that the course of development follows that expected for the normal host, *A. maculatum*. The number of melanophores is not so great as that found in the normal animals of corresponding stages of development. It will be recalled that a small number of host melanophores are typically present in an area of graft melanophores and that these host cells may well be the source of the reduced number of epidermal melanophores which are present.

Further explanation of these findings will be reserved for the discussion.

Discussion

A study of the development of cutaneous pigmentation and of the associated development of the dermis indicates that metamorphosis is only one step in the continuous process which is begun with the formation of the individual. All processes of growth and differentiation are continuous and can be divided only arbitrarily into stages. Although certain external changes which accompany the transfer from a water to a moist land habitat are fairly well demarcated, changes in the pigmentary pattern are more gradual, and dermal development is completed before metamorphosis is begun.

Recent investigations have reopened the controversy concerning the elements contributing to the development of the dermis. Detwiler (1937) found that the corium of the skin is completely developed on the side of the body from which the somites have been removed. He also followed migrating neural-crest cells, which had been vitally stained, to a position between the somites and the developing skin. From these results he was led to the conclusion that at least a part of the dermis is formed from the neural crest. Holtfreter (1935) and Raven (1936) also reached this conclusion with similar types of experiments. From the present study further evidence can be added in support of this hypothesis. The cells, which occur immediately beneath the dermis in young larvae may be those which Detwiler, in his vitally stained preparations, observed to migrate from the neural crest. This investigation has shown that the cells which lie just below the dermis in the young larva appear to become the cells of the dermis later in development. At no time is it possible to differentiate by morphological criteria the unpigmented cells of the subdermis from those of the dermis. Although morphological similarity is no indication of identical origin or of a similar fate, it may be noted that there is no difference in the cytology of the nuclei of melanophores and unpigmented cells.

Unpigmented cells occur in close association with the pigment cells which DuShane (1935) has proved to have a neural-crest origin. DuShane (1935, 1943) has shown experimentally that potential melanophores migrate in the undifferentiated state and become pigmented *in situ*. The evidence presented here indicates that at least some of the unpigmented cells of the subdermis and later of the dermis are potential pigment cells. Some differentiate before they migrate into the dermis, but the majority enter in the undifferentiated state and only later elaborate melanin. The graphs show that the ratio of pigmented to unpigmented cells is smaller in the advanced larva than it is after metamorphosis is completed. During metamorphosis the number of melanophores rapidly increases, while the unpigmented cells show a general decrease. In the adult many unpigmented cells are present among the melanophores of the dermis, and at least some of these must

represent potential melanophores. Although mitoses have been observed in differentiated pigment cells, this is not common and would contribute little to the rapid increase which takes place during metamorphosis.

Specific factors inherent in the pigment cells are of major importance in determining the pattern of distribution that is specific for the species. As has been pointed out by Twitty (1936, 1944) and Twitty and Bodenstein (1939, 1944), interspecific neural-crest grafts have shown that the melanophores of *T. torosus*, which normally form two dorsal bands, can form this characteristic pattern regardless of the environment. More recently (1945), Twitty has attempted to determine the factors present in *T. torosus* melanophores which are responsible for their aggregation at the dorsal margin of the myotomes to form the dorsal bands. From observations on explants and on transplants between *T. torosus* and *T. rivularis*, he concluded that the characteristic behavior of *T. torosus* melanophores is the result of their ability to attain a degree of differentiation higher than that observed in other species. He considers that this high degree of differentiation is necessary for the reaggregation of these cells into the dorsal bands. In the forms in which melanophores are more uniformly distributed, this high degree of differentiation is not reached.

Reciprocal graft combinations between *A. maculatum* and *T. torosus* show that, when the host and donor are the same age, melanophores of the former have a greater capacity for migration. *Ambystoma maculatum* grafts on *T. torosus* tend to be larger and to maintain themselves more clearly than do the reciprocal ones. *Triturus torosus* grafts on *A. maculatum* tend to be invaded by host cells, so that in the adult it is extremely difficult to obtain an area of pure donor melanophores. It has been observed, however, that, if older *T. torosus* grafts are transplanted to younger *A. maculatum* hosts, a larger graft area is obtained which is relatively more free of host cells. This relationship has been noted by Twitty and Bodenstein (1944), who observed that relative age differences between host and donor influence the extent of migration of neural-crest cells. In addition to these intrinsic factors, the surrounding tissues exert a definite influence upon the potentials of chromatophores. A number of workers have shown the influence of epidermis, as well as of somites and neural tube, upon the melanin-forming capacity of these cells (DuShane, 1935, 1939; Twitty, 1936; Twitty and Bodenstein, 1939; and DeLanney, 1941).

The developmental changes in pigmentation in the skin and subcutaneous regions, which have been described in this investigation, indicate that in urodeles a simple relation exists between subdermal, dermal, and epidermal melanophores. Berweger (1926) has described the development of pigmentation in *Salamandra maculosa*, and in this form she observed migration of melanophores from the cutis into the epidermis in the embryo, larva, and adult. It appears most probable, then, that all these cells arise from one cell type and that the only distinction which could be made would be one based on their position at a given time.

More recently, however, Elias (1939, 1941, 1942) has proposed an elaborate classification for melanophores, based on his discovery of certain colorless pigment cells in the dermis of the toad, *Bombinator pachypus*. The system is set forth for the amphibians in general, and he considers it to hold for urodeles as well as anurans. He recognizes four types, dis-

tinguished according to their location: (1) epidermal melanophores, found in the epidermis and occurring in all amphibians with the exception of the adults of *Bombinator* and *Hyla;* (2) adepidermal melanophores, found immediately below the marginal layer of the epidermis and occurring in urodeles and certain anurans; (3) intercutaneous melanophores, found deeper in the dermis and occurring in the adults of other anurans; and (4) subdermal melanophores, occurring in the larvae of all anurans. This scheme indicates that urodeles, throughout their life-history, possess melanophores only in the epidermis and in the dermis immediately below the epidermis and that subdermal melanophores never occur in this group. In the urodeles studied in this investigation subdermal melanophores were invariably found in the younger larvae. It is only in the older urodele larvae that melanophores are found in the dermis, while subdermal melanophores may continue to be present in reduced numbers throughout the life of the animal. Elias makes no distinction between the melanophores of the young and old urodele larva but considers that they are of only one type and occur in one position, adepidermally. This is in contrast to the condition which he describes in anurans—i.e., the degeneration of subcutaneous melanophores at metamorphosis and the new formation of dermal ones. Because of this difference, Elias homologizes the dermal melanophores of urodeles not to the dermal melanophores of anurans but to the adepidermal ones, which he found to persist throughout larval life in the discoglossides. In view of his misinterpretation of the condition existing in urodeles, one may be inclined to reject his distinction between the melanophores found in the adults of urodeles and anurans and to consider all melanophores occurring in the dermis to be of one cell type. This leaves the three types of melanophores which have been recognized here in relation to their position in the skin: subdermal, dermal, and epidermal.

A histological study of the development of the pigmentary pattern reveals that many changes take place before the onset of metamorphosis. The greatest structural changes occur before the animal leaves the water, although, grossly, little change is noted in the living animal during this time. In the species of *Ambystoma* studied, the striking color change at metamorphosis is brought about by an increase in number of melanophores and their rearrangement within the dermis to form a more complete layer instead of a loose network. Further rearrangement of the melanophores leads to the formation of spots. They are formed by the aggregation of lipophores in the epidermis and the disappearance or emigration of epidermal melanophores from these areas. Contrary to the report by Schmidt (1919) on *S. maculosa*, in *Ambystoma* some dermal melanophores do remain under the epidermis in a spot area, but they are less numerous and are more loosely arranged in the mid-dermal layer. There is no hint in the literature as to the local differences which are responsible for spotting. The melanophores themselves must contain some of the factors, since *A. maculatum* chromatophores form spots in *T. torosus* hosts, which normally are uniformly pigmented. In *T. torosus*, the pigmentary changes which occur in the dermis during metamorphosis are masked to a large extent by the heavy pigmentation of the epidermis, which develops at this time. Although the dermal pigmentary changes are of importance in forming the adult pattern, it is the epidermal pigment which plays the major role in the pigmentation of the adult in this species.

In evaluating the results of the graft series, it is of interest to compare the normal pigmentation and dermal development in the two species involved. The course of development has been shown to follow the same general trend, and comparison of the total number of melanophores (subdermal, dermal, and epidermal) and unpigmented cells (subdermal and dermal) per unit area in *A. maculatum* and *T. torosus* (Tables 2 and 3) shows that the number present at comparable stages of development is similar. However, during metamorphosis the number of unpigmented cells in *T. torosus* becomes greater than that found in *A. maculatum*. From these data it is impossible to state what difference is a significant one; hence it can be said only that the value in the adult *T. torosus* is about one-third larger than that found in *A. maculatum*.

Counts of the total number of melanophores (subdermal, dermal, and epidermal) and unpigmented cells (subdermal and dermal) per unit area in the two graft series (Table 4) show that the course of development is very similar. No conclusion can be drawn with regard to the source of the elements contributing to the dermis, since the numbers found may be considered normal for either species and the melanophores maintain themselves as well in the graft as in the normal environment. If it is assumed, as has been suggested above, that the unpigmented cells, at least a part of which are potential pigment cells and which participate in the formation of the dermis, are derived from the neural crest, then it is seen that these cells are likewise not adversely affected by the strange environment of the host. However, in the case of the metamorphosing and the adult *T. torosus* with *A. maculatum* grafts, the number of melanophores is far in excess of that found in the normal animals of this series. It is possible that this is partially due to the inability of donor melanophores to penetrate the epidermis of the host where they would normally give off their pigment and so become depleted. The suggestion is made that the donor melanophores accumulate in the dermis, and this results in the increased number. Still another factor may be a difference in threshold of reactivity of the two types of melanophores. *Ambystoma maculatum* melanophores may be responding to a higher level of melanogenic substance in the *T. torosus* host. The latter is the least probable, however, since it is known that the tissues of *T. torosus* are relatively poor in the ability to promote melanin formation (DeLanney, 1941). Further work is needed to bring out these relationships.

Attempts have been made in these observations to determine the origin and formation of epidermal pigment. Although epidermal melanophores do not develop in the absence of the neural crest, DuShane (1935) states that decisive evidence of their source is lacking. No morphological difference was present in the epidermal melanophores of the different species that he used, so xenoplastic grafts throw no light on their origin. The fact that dermal melanophores are the first to develop and that they are so commonly seen migrating into the epidermis gives support to the hypothesis that epidermal as well as dermal melanophores are derived from the neural crest. Migration of dermal melanophores into the epidermis occurs throughout the larval period and is particularly prominent in the advanced larva and during metamorphosis in *T. torosus*, at the time when epidermal melanophores increase most rapidly. The evidence seems to indicate that the morphology of melanophores may be modified by the epidermal environment.

After metamorphosis, epidermal melanin granules in *T. torosus* become finer, while epidermal melanophores become more finely branched and contain finer granules. It appears that these changes take place in the melanin which is already present in the epidermis, since there is no indication that this peculiar melanin is brought to the epidermis from elsewhere at this time. The epidermal environment may also modify the morphology of epidermal melanophores, and, as a result, it may not be possible to judge the source of these cells by their morphology.

DuShane (1938) has shown that in animals with extirpated neural crests, dermal melanophores migrated into the unpopulated trunk region before epidermal melanophores appeared. This is the time-relation expected if epidermal melanophores have their origin in the neural crest, as do the dermal ones.

The significance of the melanin that occurs in the epidermal cells has been interpreted in a variety of ways. In some cases it has been confused with egg pigment and so has been considered a product of metabolism. Berweger (1926), in her study on *S. maculosa*, has never observed the penetration of melanophore processes into epidermal cells and has frequently found epidermal pigment in areas in which no chromatophores occurred. She concludes, therefore, that epidermal cells have the capacity to form melanin independently of the presence of melanophores. Few investigators have considered this melanin to be derived from the melanophores, which extend their processes for long distances between the epidermal cells (e.g., Ehrmann, 1885) and, in the cases observed here, without any doubt do enter these cells. The present observations have shown that there is a close correlation between the presence of epidermal melanophores and melanin in epidermal cells. In the species studied here, epidermal pigment occurred only in regions where epidermal melanophores could be found. If the melanophores are greatly contracted, it is unusual to find much epidermal pigment, while, when they are expanded and their processes penetrate the epidermal cells, a great deal of melanin may be present in these cells.

The neural-crest graft series supply the best evidence of the relation between melanophores and pigment in the epidermis. Observations on these series make it evident that the donor melanophores do not migrate into the epidermis of the host and that in their absence no melanin is present in the epidermis. As a result, neither epidermal melanophores nor epidermal pigment occur in the epidermis covering an area containing only graft chromatophores. It appears that these pigment differences result entirely from factors present in the epidermis, since an area of graft epidermis adjacent to the unpigmented host epidermis contains the normal amount of melanin. These results strongly indicate a common origin of dermal and epidermal melanophores and make highly probable a relation between epidermal melanophores and the pigment in epidermal cells. It must be understood, however, that these statements are made only for the species which have been investigated; generalizations must await descriptions of the conditions which hold in a wider variety of amphibians.

Studies of other classes of vertebrates have revealed that the relation between melanophores and epidermal pigment which has been suggested here is not peculiar to amphibians. In birds it has long been known that the melanophore releases its melanin granules to the barb and barbule cells of the developing feather (e.g., Aeby, 1885; Strong, 1902; Willier and Rawles, 1938, 1940; and,

most recently, Watterson, 1942). It is by this release of pigment from the chromatophore that the feather becomes pigmented. The pigment cell then degenerates so that the entire pigmentary function in the adult is taken over by the feathers. Here, then, there is a greater specialization in the function of the epidermal melanophores than that found in amphibians. It has been shown, however, that there is a reservoir of potential pigment cells, which differentiate in the development of regenerating feathers (Foulks, 1943).

In mammals recent work indicates that melanophores may supply pigment to the epidermal cells of the developing hair follicle. Rawles (1940) took pieces of ectoderm with adhering mesoderm from over the neural crest, as well as from the limb buds, of 8½–12-day mouse embryos. When transplanted to the coelom of White Leghorn host embryos, all grafts developed skin and hair follicles; but grafts from the hind-limb bud contained no pigment cells in the follicles and the hairs were entirely without pigment. Aeby, as early as 1885, stated his belief that epidermal pigment always came from chromatophores and that the epidermis was unable to form pigment in any class of vertebrate.

In birds the origin of epidermal pigment from melanophores has been clearly demonstrated. The same relationship appears to hold in mammals, although as yet it has not been so conclusively demonstrated. In amphibians indisputable proof on this point is still lacking, but in the light of evidence in the other classes of vertebrates it would not be surprising to find the same relation.

Summary and Conclusion

The development of pigmentation and of the dermis is described in three species of urodeles—*A. tigrinum*, *A. maculatum*, and *T. torosus*. The changes in the dermis and in the pigmentation in *A. maculatum* and *T. torosus* with reciprocal neural-crest grafts are given special consideration, as well as the problem of the source of epidermal pigment.

The development of the dermis

1. In the young larva the dermis consists of a thin layer of collagenous fibers which contains no cells. As development proceeds, the dermis increases in thickness and soon contains unpigmented cells and melanophores. The cells apparently originate by migration from the subdermis. Subdermal cells are most numerous just before dermal cells appear. As dermal cells increase in number, the cells of the subdermis become less numerous.

2. Skin glands begin to form in the advanced larva and are large and well differentiated when metamorphosis is completed. The striking increase in dermal thickness which occurs in the advanced larva is associated with the rapid increase in the size of these glands.

3. The unpigmented cells of the subdermis and dermis resemble undifferentiated mesenchyme cells. From indirect evidence it seems certain that at least some of these cells are melanoblasts, and it has been shown that they contribute to the formation of the dermis. Since melanoblasts are known to have a neural-crest origin, the origin of the other cells of the dermis from this source also is highly probable. The neural-crest graft series give no information concerning the origin of the dermis, since the mode of development is similar in the two species which were employed (*T. torosus* and *A. maculatum*).

THE DEVELOPMENT OF PIGMENTATION IN THE DERMIS

1. In the young larva, melanophores are found below the dermis and in the epidermis. Later in larval development they occur in the dermis also, apparently having migrated from the subdermis. During metamorphosis and in the adult, melanophores are rarely found in the subdermis but occur principally in the dermis and the epidermis.

2. In the two species of *Ambystoma*, changes in the external appearance of the pigmentary pattern which occur at metamorphosis result from an increase in number of dermal melanophores and their aggregation below the epidermis to form a fairly complete layer. This results in the black background which is characteristic of these species. Spots result from the aggregation of lipophores in the epidermis and the slight decrease in number of melanophores in the underlying dermis.

3. In *T. torosus*, metamorphosis is characterized by the development of a uniform brown pattern. This is brought about by a large increase in number of epidermal melanophores and melanin during metamorphosis and a contraction of dermal melanophores.

4. Grafted melanophores are capable of maintaining themselves in the environment of a foreign species and of reproducing the donor pattern.

THE DEVELOPMENT OF EPIDERMAL PIGMENTATION

1. Epidermal melanophores have highly branched processes, which extend for long distances between the epidermal cells. At their ends the processes can enter the epidermal cells, where they pass over the outer side of the nucleus and appear to break up. In animals with interspecific neural-crest grafts, donor melanophores appear to be unable to migrate into the epidermis of the host. No melanophores or epidermal pigment are present in the epidermis overlying these areas of donor melanophores.

2. The intimate relation between melanophores and epidermal cells suggests that melanophores are the source of the melanin that occurs in the epidermis. This relation has been shown to be true in birds, and the evidence in mammals, although not so conclusive, is strongly suggestive of the same conclusion.

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PLATE I

The development of the skin in *A. tigrinum*. Outlines with camera lucida, details diagrammatic. Magnification *ca.* ×330. *A*, 24-mm. larva; *B*, 58-mm. larva; *C*, 73-mm. larva; *D*, 135-mm. adult. Abbreviations: *D.*, dermis; *D.M.*, dermal melanophore; *D.U.C.*, dermal unpigmented cell; *E.*, epidermis; *E.C.*, epidermal cell; *L.C.*, Leydig cell; *M.*, melanophore; *S.G.*, skin gland; *S.M.*, subdermal melanophores; *S.U.C.*, subdermal unpigmented cell.

PLATE II

A, cross-section of *T. torosus* embryo, stage 44. The melanophores (*M.*) form a network, sometimes more conspicuous segmentally, over the dorsal margins of the myotomes. Grossly, they appear to form two longitudinal bands because of the deeper position of the melanophores located in the mid-line. *Ca.* ×80.

B, section of skin of adult *T. torosus*, showing the large amount of melanin in the epidermal cells and the numerous epidermal melanophores, as compared to the species of *Ambystoma*, and the highly contracted dermal melanophores. *Ca.* ×295.

C, section of skin of adult *A. maculatum* with grafted *T. torosus* neural crest. Section includes some donor epidermis and shows the large amount of pigment in these cells. The host epidermis is entirely lacking in pigmentation. Dermal pigmentation is distinctly reduced from that found in the normal *A. maculatum*. *Ca.* ×295.

D, section of skin of adult *T. torosus* with grafted *A. maculatum* neural crest. Section includes some donor epidermis which contains a small amount of pigment, but much less than is found in the normal *T. torosus* epidermis. Dermal pigmentation is greatly increased over that found in the normal *T. torosus*, and the melanophores are highly expanded. *Ca.* ×295.

E, section of skin of an advanced larva of *T. torosus*. Epidermal pigmentation has just become heavy at this stage, and epidermal melanophores are highly expanded. Their processes extend for long distances between the epidermal cells and send off branches which enter the epidermal cells. *Ca.* ×295.

Abbreviations: *D.*, dermis; *D.M.*, dermal melanophore; *D.U.C.*, dermal unpigmented cell; *E.*, epidermis; *E.M.*, epidermal melanophore; *G.E.*, graft epidermis; *H.E.*, host epidermis; *M.*, melanophore; *S.G.*, skin gland; *S.U.C.*, subdermal unpigmented cell. Outlines drawn with camera lucida, details diagrammatic.

HEAT DEATH, HEAT INJURY, AND TOXIC FACTOR[1]

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WHEN a cell is exposed to excessive heat, it dies. But in a comlicated organism all types of cells are obviously not equally sensitive to heat; and in such an organism, if certain cells die before others, they are likely to produce toxic substances which will have a deleterious effect on other cells not so sensitive to heat. Thus, on purely a priori grounds, it is logical to assume that, when any metazoan animal is exposed to heat, toxic substances would be produced by the cells which died first and that these toxic substances would have a harmful, perhaps a lethal, effect on the whole animal. Obviously, also, in those animals with a well-developed circulation the possible effect of such toxic substances would be enhanced.

From the standpoint of practical medi-

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PLATE I

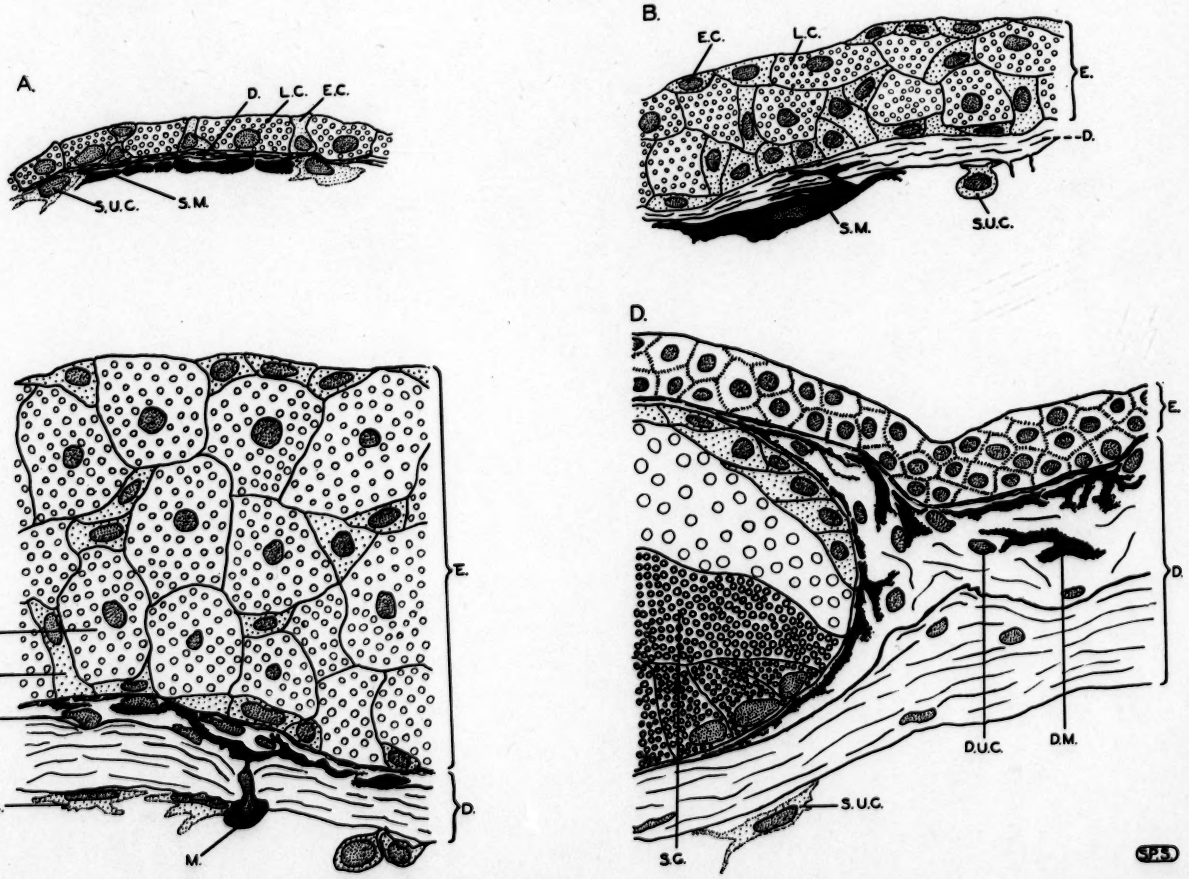
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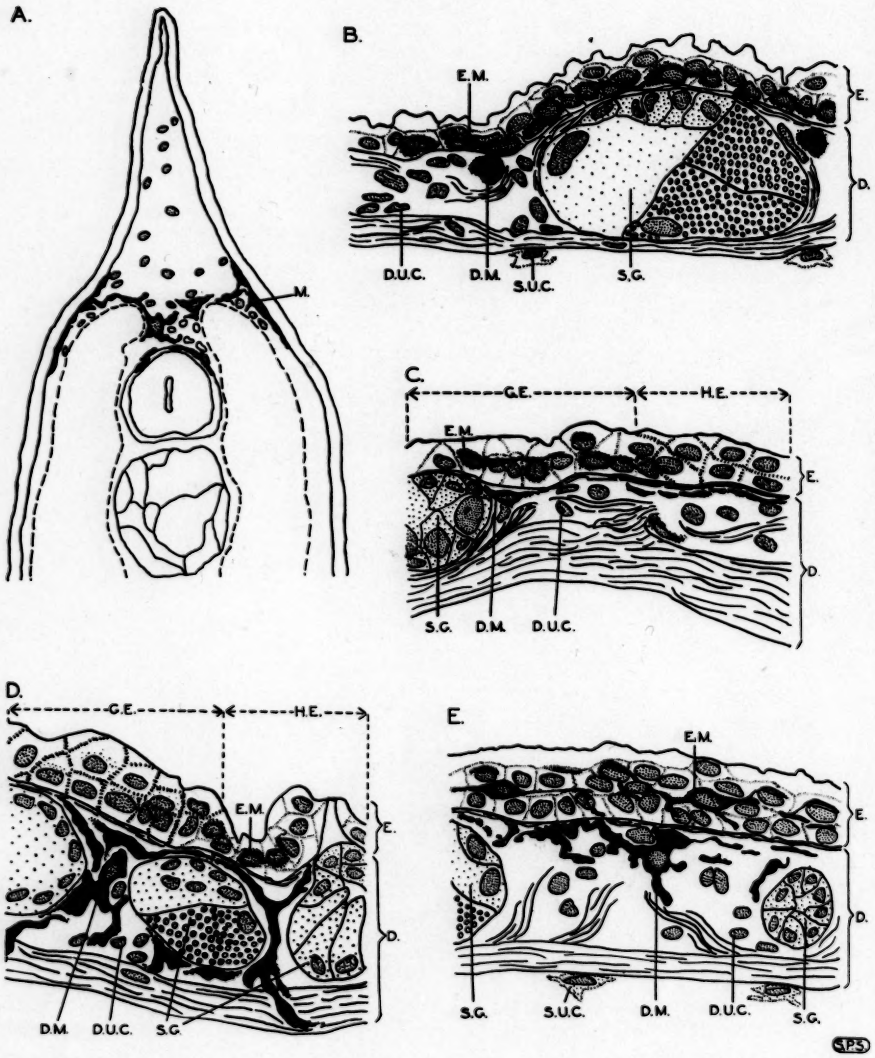
PLATE II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cine, the subject has great importance. Ever since man discovered fire, burns have been one of the commonest of human ailments. The literature on burns and burn therapy is vast (Harkins, 1942, 1945). Many of the authorities in this field believe that in burn injury one of the primary factors is the presence of toxic substances, the so-called "toxic factor." Not only burns but also other types of cell or tissue injury produce effects which are harmful or lethal to the entire animal. Severe injuries, such as are produced by battlefield wounds, by automobile accidents, or by other hazards of our modern industrial civilization, frequently cause death. Impairment of the circulation, which can be initiated by tying off the legs of a rat or a rabbit, can apparently produce toxic substances; for, when the circulation is restored by removal of the ligature, the rat or rabbit commonly dies. In all these cases in which injury of a part of an animal causes death of the whole organism, the symptoms which precede death are to some extent similar. Typically, the arterial blood pressure drops, and the volume of the blood decreases. These are aspects of a condition known as "shock," but the exact definition of shock is uncertain. In addition to the shock produced by burns, by trauma, or by impairment of the circulation for a time with a ligature, shock may likewise be caused by excessive hemorrhage; this is perhaps also a consequence of a diminished or impaired circulation. A type of shock may likewise follow as a consequence of an anaphylactic reaction. So much has been written about shock by medical research workers that an entire science has been built up (see, for example, Moon, 1942; Wiggers, 1942).

Unfortunately, it is not a very satisfactory science. Basically, there are two main types of explanation which have been advanced to explain shock. According to one group of workers, the primary effect of tissue injury is the loss of fluid from the blood. This is the "fluid-loss" theory. On the other hand, the "toxic-factor" theory maintains that the primary factor in shock is a toxic substance or substances produced by injured tissues. The two types of theories are not mutually exclusive. It is certain that the loss of fluid which occurs when tissues are burned or otherwise injured is a factor in producing shock. But it seems equally certain that this cannot be the whole explanation. Thus Haist and Hamilton (1944) have shown that, if the hind legs of rats are tied off and then the ligature is removed, the fluid loss from the blood is complete within an hour. If after an hour the ligature is renewed, the animals do not die; whereas if the legs are left permanently unligatured, the animals do die. There is, therefore, rather clear evidence that in this type of shock some toxic factor is involved. Other recent authors have supported this view (cf. Shorr, Zweifach, and Furchgott, 1945).

Toxic factors are probably also involved in a type of injury in which the term "shock" is not ordinarily used. Excessive treatment with Roentgen radiation causes general effects on animals and human beings, and the effects are not confined to the region irradiated.

One of the difficulties with the toxic-factor theory of shock is the fact that no one has with any certainty isolated definite toxic substances from the blood of injured or shocked animals. It is true that toxic substances may be obtained from various types of tissue extractions; but that these substances actually play a role in the intact injured animal has never been proved.

PRELIMINARY STUDIES ON THE EFFECT OF HEAT ON LABORATORY ANIMALS

The common laboratory animals—mice, rats, rabbits, and guinea pigs—are

rather sensitive to heat and die quickly if exposed to temperatures of 40°–50° C. This is shown in Table 1. In this table the number of animals used in a given experiment is shown by the figures in parentheses. The experiments were performed in a room kept at constant temperature. The humidity of this room stayed rather consistently at about 15 per cent. In the experiments reported, the animals were not allowed to have contact with water. If animals are allowed to have such contact, the survival time, in the case of the rats at least, is lengthened and very variable, for the animals splash about in the water and thus are, to some extent, cooled.

TABLE 1

AVERAGE SURVIVAL TIME OF ANIMALS EXPOSED TO HEAT

(In Minutes)

Conditions	Temperature (°C.)					
	40	42–43	44	45	48	49–51
Rats in the hot room	213±36 (6)	144±12 (36)		79±7 (16)		31±1 (36)
Rats in water bath to knees				154±7 (55)		
Guinea pigs in the hot room					100±4 (11)	54±5 (4)
Guinea pigs in water bath to axilla			69±2 (18)			

Rodents are much more sensitive to heat than are certain other mammals. A calf which was placed in the hot room at 52° C. lived for 5½ hours, and two cats lived for over 8 hours at 50° C.

Rats exposed to lethal temperatures in the hot room show the following symptoms. Almost from the start there is evidence of muscular weakness; the animals drag themselves along the floor, and their hind legs do not seem capable of supporting the weight of the body. The testes descend; they lie on the floor and are pulled along after the rats as they move. Breathing movements are greatly exaggerated. As death approaches, a characteristic clicking noise appears. This noise seems to originate in the bronchial passages. A moment or two before death, it is hard to obtain blood from the peripheral circulation; and, even when a cannula is placed in the femoral artery, hardly any blood emerges. Nevertheless, the ears are red, so that obviously the supply of blood to the skin is not impaired. Just before death, convulsions occur.

If a heat-killed rat is autopsied immediately after death, the right side of the heart is seen to be greatly distended. The lungs show emphysema, and there is some evidence of fluid loss from the lungs. (In these observations on the gross pathology of heat-killed rats, we were fortunate to have the help of Dr. D. R. Coman, of the Department of Pathology of the University of Pennsylvania.)

HEAT DEATH AS A RESULT OF PARTIAL EXPOSURE

If toxic substances play a part in the heat death of an animal, then it should be possible to produce death by exposing just a part of the animal to the heat. We

did numerous experiments on rats exposed only partially to heat. In these experiments the rats were tied into a confining, narrow, wire enclosure, and their hind legs were immersed up to the knee joint in water kept at a constant temperature of 45° C. Under these conditions convulsions are not readily observed; but apparently the animals die in essentially the same manner as they do in the hot room, although they survive for a somewhat longer time (see Table 1). The rectal temperature of these rats killed by immersion of their legs in hot water is not very high. At death the average rectal temperature was 38°.1 ± 0°.4 C.[2] Thus it is clear that the death of these animals is not due to hyperthermia. Apparently, it is due to the formation of a toxic substance or substances in the heat-exposed regions. This is borne out by the fact that death can be prevented by the application of a tight ligature around the legs of the rats above the region of immersion. The ligatures prevent the passage of toxic substances from the legs to the rest of the animal; but the ligature experiments do not furnish conclusive proof that toxic substances are involved, for it is possible that heating of the leg tissues causes a breakdown of blood-vessel walls and escape of fluid and that this fluid loss is to a large extent prevented by tying off the circulation. Later experiments, however, indicate that a toxic factor or factors are really involved.

In our opinion the heat death of rats in the hot room is not essentially a different phenomenon from the death produced when the legs are immersed in hot water. In the leg-immersion experiments we are really dealing with burns, even though the temperature of the water is not very high. Thus in our experiments, apparently, there is no fundamental difference between heat death and death resulting from burns. It should be remembered that in these experiments with animals exposed to the heat of the hot room, we were dealing with animals which have essentially no sweat glands and are scarcely capable of sweating. Thus, although they lose a large quantity of water from the lungs, there is little, if any, loss of chloride other than that which may occur through the urine (or the intestine).

Rats appear to be especially sensitive to heat death and to death from burns. On the other hand, guinea pigs are not so sensitive to burns. If their feet and legs are immersed in hot water (44° C.), they do not die. Death occurs only if the animals are immersed up to the axilla. The average death time for animals subjected to this type of exposure was 69 ± 2 minutes.

In the experiments on rats, the legs of which were exposed to heat, we were led to inquire whether any effective antidote could be found for the toxic substance(s) which presumably was causing the death of the animals. We tried injecting various substances intraperitoneally into these burned rats in the hope of finding something which would markedly favor their their survival. No great success was obtained, but injection either of sodium chloride in isotonic solution or of Ringer's solution often had a favorable effect.

RELATIVE SENSITIVITY OF NERVE AND MUSCLE TO HEAT

If it is true that the burned leg of a rat produces a toxic substance or substances, then presumably some of the tissues of the leg are more sensitive to heat than others and would be more likely to yield the toxic material. We were led to inquire, therefore, into the relative sensitivity of different tissues. Apparently, muscles are more readily killed by heat

[2] All ± signs designate standard errors.

than are nerves. In the experiments with rats we found that, after the legs had been immersed in hot water for a couple of hours, the muscles became incapable of direct electrical stimulation. And yet the sensory nerves of the legs still remained functional; for, when the feet were stimulated electrically, the animals showed signs of pain and such indications of pain continued for some time after paralysis had set in.

In isolated nerve-muscle preparations of the frog, it can readily be shown that nerve is more resistant to heat than muscle. As a matter of fact, this was well known to Claude Bernard (see Bernard, 1876; also Stiles, 1864). Experiments were carried out in our laboratory by L. N. Sokoloff and E. W. Tobia. The gastrocnemius-sciatic preparation was used, the muscle alone or the nerve alone being immersed in hot Ringer's solution. The immersed muscle was stimulated directly and also indirectly through the attached nerve. The results showed clearly that the frog nerve is able to withstand heat for a longer time than the muscle. Also it is evident that the muscle loses its ability to respond to indirect stimulation before it loses its ability to respond to direct stimulation; the loss of response is irreversible. This may mean that the myoneural junction dies first; or it may merely indicate that direct stimulation, inasmuch as it can affect all parts of the muscle fibers, is more efficient in causing a recognizable contraction in partially killed muscle.

THE ACTION OF MUSCLE EXTRACTS

The leg of a rat consists very largely of muscle; other tissues are, of course, present, but they constitute a relatively small percentage of the entire mass. If muscles are especially sensitive to heat and if the death of the whole animal is occasioned by the liberation of some substance or substances from the heat-affected tissues, it might be expected that extracts from heated muscle would have a toxic effect and that this effect would be related to heat death and heat prostration. Clearly, therefore, it became of interest for us to inquire into the possible toxicity of extracts of heated muscle.

The first experiments were done with extracts made from frog muscles. Isolated muscles from a number of frogs were heated to approximately 50°–60° C. for 3 hours in Ringer's solution. An equal weight of normal muscles was soaked for a similar time in Ringer's solution at room temperature. Paired sartorius muscles from frogs were then exposed to these solutions at 38° C. Survival time was determined by loss of response to an electrical stimulus. Part of the data is given in Table 2 (the rest of the data, not presented here, is entirely consistent with the results shown in the table). The experiments show rather clearly that from heat-killed frog muscle a substance (or substances) may be extracted which hastens the heat death of other frog muscle. It is possible that the substance may also be extracted from normal muscle; but, if this is so, then much smaller amounts of the substance(s) would be given off by the uninjured muscle. The toxic substance(s) from frog muscle is dialysable (through Cellophane) and is sufficiently heat stable so that it can be boiled to dryness. However, the toxic substance(s) will not withstand ashing (over a Meker burner). We also tested the effect of the extracts of heat-killed muscles on the survival of whole frogs at 38° C. A dialysate of an extract of heat-killed muscle, when injected into the lymph sac of frogs exposed to heat, very markedly decreased the survival time of such animals, either in comparison with frogs injected with Ringer's solution or in comparison with frogs injected with a

dialysate of an extract obtained from normal muscles.

Following these early experiments with frogs, more extensive experiments were made with mammalian muscle. Muscles from rabbits or from guinea pigs were cut into small pieces, and these were then extracted in distilled water. Extracts made with hot water and with cold water were compared. In general, the extracts from mammalian muscle resembled those from frog muscle. When the water extracts were dialyzed, the toxic substance(s) was found to pass through the Cellophane membrane of the dialysis sack. In order to make suitable comparisons of different types of extracts, a standard procedure was adopted. A known weight of muscle was extracted for several hours with several times its weight of distilled water. Toluene was added to prevent putrefaction. The extract was dialyzed through a Cellophane sack, which was placed in several changes of distilled water. The water was then evaporated and the resultant material taken up in a known volume of distilled water. This solution could then be tested for its effect on the heat death of muscles, or it could be injected intraperitoneally into a rat or mouse subjected to the heat of a hot room. Two important facts soon became evident. First, the extracts from all sorts of animals behaved in similar fashion. We studied extracts from the muscles of frogs, rats, guinea pigs, rabbits, cats, dogs, and calf. Second, it was not necessary to expose the test animals to the heat of a hot room. Thus rats, given sufficiently strong injections of extracts from heat-killed muscles, behaved almost exactly like the rats exposed to the lethal temperatures of the hot room. These rats, injected under ordinary room-temperature conditions, showed the same muscular weakness, the same exaggerated breathing movements, and, as death approached, the same clicking noise previously described for the rats killed in the hot room. A moment or two before death the injected animals typically went into violent convulsions. Autopsy again showed great distention of the right side of the heart. Moreover, rats killed by in-

TABLE 2

AVERAGE SURVIVAL TIME OF PAIRED SARTORIUS MUSCLES EXPOSED TO 38° C. EITHER IN AN EXTRACT OF NORMAL MUSCLE IN RINGER'S FLUID OR IN AN EXTRACT OF HEAT-KILLED MUSCLE IN RINGER'S FLUID

Number of Pairs	Treatment of Extracts	Survival Time in Extract of Normal Muscle (In Minutes)	Survival Time in Extract of Heat-killed Muscle (In Minutes)
14	Whole extract	50	26
19	Dialysate fraction	91	53
5	Nondialysate fraction	99	97
15	Dialysate fraction, boiled	86	46
8	Dialysate fraction, boiled to dryness and redissolved	93	50
6	Dialysate fraction, ashed and redissolved	58	58
10	Dialysate fraction, treated with activated charcoal and filtered	103	55

jection of muscle extracts had but little blood in the peripheral circulation. There was one point of difference between the injected rats and those exposed to heat: the injected rats showed a lack of blood in the ears, whereas in the rats in the hot room the ears were red.

In evaluating our work with muscle extracts, quantitative comparisons are necessary. The method of comparison that we used first was to express the strength of an extract in terms of the number of grams of tissue required to produce a given effect. Thus, if the death of a 100-gm. rat was produced by the extract obtained from 20 gm. of muscle (but not from less), this value of 20 was taken as a measure of the extract's potency. In this scheme the stronger an extract, the smaller the number representing its potency. This tended to complicate our thinking; and, accordingly, we decided to use, as a measure of potency, the reciprocal of the minimal number of grams of tissue the extract of which would kill a 100-gm. rat when injected intraperitoneally. Such a reciprocal value can be called a "rat (death) unit," and we shall define as a rat unit the amount of toxic substance necessary to kill a 100-gm. rat. The scale of the unit is determined by taking the reciprocal of the number of grams of tissue represented in the extract and multiplying this value by 1,000. This gives the number of rat units per kilogram of tissue. In our original example, if a lethal quantity of extract represented the total extract from 20 gm. of tissue, the number of rat units per kilogram of tissue would be 50. In testing the extracts it is not necessary always to use rats weighing 100 gm. When proportionally smaller amounts are injected into smaller rats, reasonably consistent results are obtained. Also mice can be used as test objects instead of rats. Indeed, as noted previously, the toxic substance(s) is not species specific. It is essentially immaterial whether a rat is injected with extract from rat muscle, rabbit muscle, or guinea-pig muscle. Dog muscle extracts and those from calf muscle act in the same way.

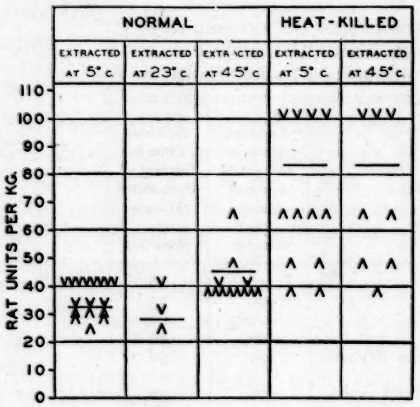

FIG. 1.—Toxicity of extracts of normal and heat-killed rabbit muscle.

Results with rabbit muscle are shown in Figure 1. In this each individual *V* and each inverted *V* represents a single test with a given quantity of extract. The apex of each symbol is to be correlated with the scale of unit values at the left. A *V* indicates that in a given test the animal did not die and that therefore the extract being tested did not contain so many rat units per kilogram as is shown in the ordinate scale at the left. An inverted *V* indicates that, for the test in question, the animal did die and that therefore the extract tested contained more than the number of rat units per kilogram shown in the ordinate scale at the level of the apex of the symbol. An inverted *A* indicates that in a given test the animal was close to death and that therefore the correct assay is only slightly less than the point indicated. The estimated assay level is indicated by a horizontal line.

Inspection of Figure 1 shows that hot-water extracts of rabbit muscle are more potent than cold-water extracts; that is to say, they contain more rat (death) units. It is to be noted also that, if a rabbit is killed by exposure to the heat of the hot room and its muscles are then extracted with water, the resultant extracts show a high level of toxicity, whether the extraction occurred in hot or cold water. Nor does there seem to be any difference between the hot- and cold-water extracts of the muscles of heat-killed rabbits.

Our experimental study of the extracts was not limited to intraperitoneal injections. (Animals are much more sensitive to intravenous than to intraperitoneal injections.) We injected muscle extracts into the marginal vein of the ears of heparinized rabbits, anesthetized with urethane. Kymograph records show that, following injection of a small amount of an extract, there is a sharp fall of blood pressure. However, following the injection of a sublethal dose, the rabbits become less sensitive to subsequent doses, so that eventually they are able to withstand injection of amounts of the extract which would originally have been fatal. For example, one rabbit was not killed by an injection of 18.4 ml. of an extract for which the minimum lethal dose was approximately 4.2 ml. The injection of the 18.4 ml. was made intermittently over a period of 21 minutes, with increasing dosage per injection. The final dose, which did not kill the rabbit, was one and a half times the minimum lethal dose.

EXTRACTS OF VARIOUS TISSUES

Muscles are not the only tissues of the body which can provide a source for toxic substances. Indeed, when various organs are extracted, toxic substances may be obtained in the same way as from muscle, and these extracts have the same effects on rats as do the muscle extracts.

A calf was killed in our hot room by exposing it to a temperature of 52° C. Its organs were then extracted, with the standard procedure already described for muscle. Results are shown in Figure 2. The symbols are the same as those used in the previous figure. Heart and tongue, both consisting essentially of muscle, have a higher content of toxic substance(s) than do other organs tested.

Figure 3 shows results obtained with the organs and tissues of a scalded dog. For this animal we are grateful to Drs. Lockwood and Parkins, of the Department of Experimental Surgery of the University of Pennsylvania. The dog was anesthetized with nembutal, its hair was clipped, it was immersed up to the axilla in water at a temperature of 82° C., and it was exposed to this temperature for 60 seconds. Various organs and tissues were then removed and extracts made from them. For comparison, skin and muscle were taken from a control dog, not scalded. In the case of the dog, as in the calf, more toxic substance(s) can be obtained from muscle (per gram) than from any other part of the body tested. An interesting fact is that the skin yielded a very small amount of toxic substance, and the one test tried indicated that this amount was no greater in the scalded animal than it was in the control. On the other hand, the muscle from the scalded dog yielded almost twice as high a concentration of toxic substance(s) as did the muscle of the unscalded animal.

TOXIC FACTOR(S) IN BLOOD

The fact that toxic substances may be extracted from the tissues and organs of animals is not very surprising in the light of the older literature on tissue extracts. There is, indeed, an old and many-sided literature on the subject; some of it is

reviewed by Moon (1942). More recent references are given by Shorr, Zweifach, and Furchgott (1945). But, although it is easy to prove that simple extracts from heat-killed tissue contain a toxic substance or substances, it is not so easy to show that such substances actually play a part in the death of an animal exposed in whole or in part to excessive heat. Does burned tissue actually liberate toxic factor(s) into the blood of a heat-injured or burned animal and can such toxic factor(s) be demonstrated there? This is rather a crucial point in the whole theory of toxic factor(s), not only in burn-shock but in other types of shock as well.

In experiments done in the early part of 1944, we found that, when a rabbit was killed in our hot room or was exposed to heat almost sufficient to kill it, the blood of such an animal could be demonstrated to contain toxic factor(s). Such toxic factor(s) could not be extracted from normal blood. The experimental data are shown in Figure 4. The symbols used in this figure are the same as those used in the earlier figures and described previously. In order to obtain extracts containing toxic factor(s), the defibrinated plasma was dialyzed in a Cellophane sack, the dialysate boiled to dryness, the residue extracted with a small volume of

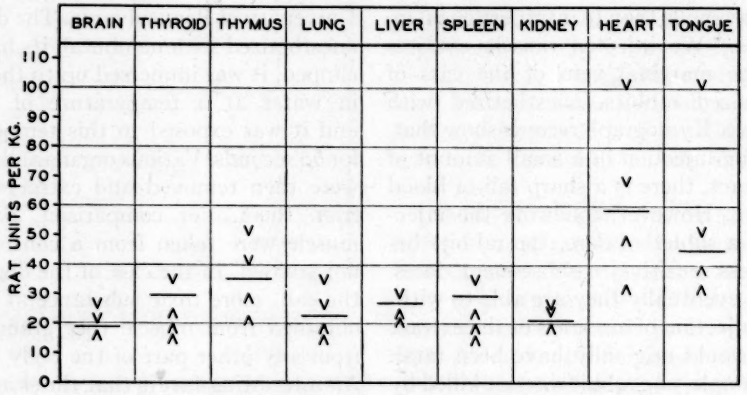

FIG. 2.—Toxicity of extracts of various organs of a heat-killed calf

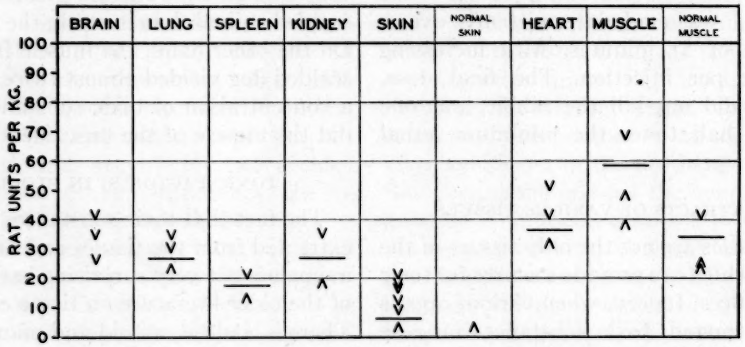

FIG. 3.—Toxicity of extracts of various organs and tissues of a scalded dog

methyl alcohol, the alcoholic extract boiled to dryness, and the residue redissolved in distilled water. Extracts from the blood of normal animals showed no evidence of toxicity; those from the blood of heat-killed animals were highly toxic, provided that the extract was made from defibrinated blood and not from blood that had been allowed to clot. Figure 4 also gives data on six experiments with rabbits whose legs had been ligatured. In four cases, removal or the ligature resulted in a condition of mild shock, and the blood from these animals showed presence of some toxic factor(s). In two other experiments, in which removal of the ligature resulted in severe shock, the results were somewhat divergent; in one case, much more toxic factor(s) was present than in the other.

The blood of scalded dogs was also found to contain toxic factor(s). Some of the data are shown in Figure 4. In addition to the dog previously mentioned, two other dogs were also scalded for us by Dr. Parkins, in the laboratory of the Department of Experimental Surgery of the University of Pennsylvania. These dogs were anesthetized, their hair was clipped, and they were immersed up to the axilla in a water bath kept at a temperature of 86° C. Exposure was for 60 seconds. In one experiment, at various intervals following exposure, blood was taken from the dog by Dr. Parkins. We then defibrinated it, centrifuged it, and dialyzed the serum in a Cellophane sack. The distilled water of the dialysate (containing toxic factor[s]) was then boiled to dryness, and the methanol-soluble fraction

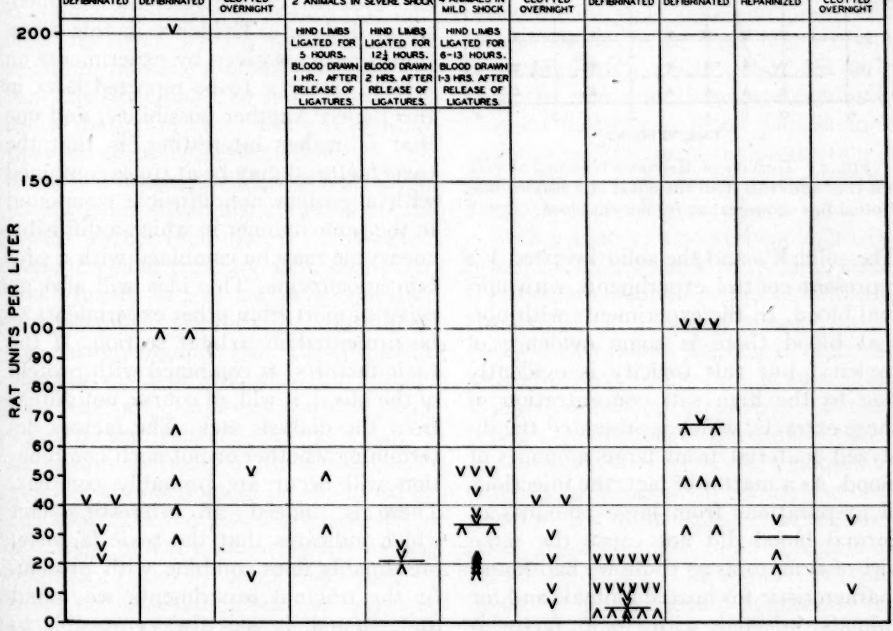

FIG. 4.—Toxicity of dialysates obtained from the blood of animals subjected to shock-producing treatments

of the residue was tested for toxic factor(s) in the manner previously described. Within an hour after scalding, the blood showed 67 rat units per liter. The full record of the experiment is shown in Figure 5. As before, each individual *V*, each inverted *V*, and each inverted *A* represents a single test of a certain amount of concentrate for a given sample, and the symbols are to be interpreted as before. Because of the small amount of blood available, tests were made with mice rather than with rats.

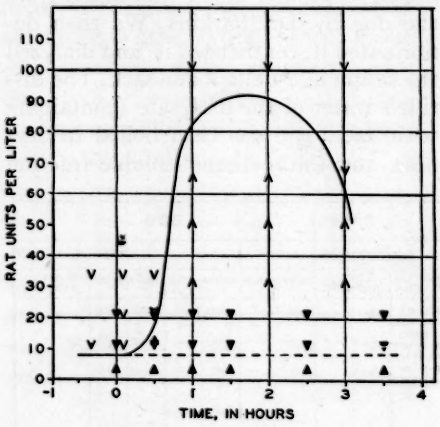

FIG. 5.—Toxicity of dialysates obtained at various time intervals from the blood of a scalded dog. Dotted line shows values for normal blood.

The solid *V*'s and the solid inverted *V*'s represent control experiments with normal blood. In the experiments with normal blood there is some evidence of toxicity; but this toxicity is evidently due to the high salt concentration of these extracts, which represented the dialyzed material from large amounts of blood. As a matter of fact, the injections of preparations from large amounts of normal blood did not cause the same sort of symptoms as those we had found characteristic for heated animals and for animals injected with toxic factor(s) from heated or burned animals.

In later experiments Morris J. Frumin attempted to repeat the results that we had obtained with heat-killed rabbits and scalded dogs. Unfortunately, he was never able to obtain any evidence for the existence of toxic factor(s) in the blood of the heat-injured animals. The reasons for Frumin's failure are not clear. We thought, at first, that there might be some difference in the Cellophane dialysis sacks used in the earlier and later experiments. However, Frumin tried various samples of Cellophane, without result. His method of concentrating the dialysate differed slightly from the method we had used previously in that there was a delay of some few hours before concentration was begun. This may be significant.

Apparently, if toxic factor(s) is present in blood, it is not always a simple matter to isolate it. For one thing, the factor(s) may be lost in the boiling process used to concentrate the distilled-water dialysate. Evidence in this direction is perhaps given by experiments on muscle extracts, to be reported later in this paper. Another possibility, and one that is rather interesting, is that the toxic factor(s) may be at times combined with a protein nondiffusible compound in the same manner in which a diffusible coenzyme may be combined with a protein apoenzyme. This idea will also receive support from other experiments to be presented in a later section. If the toxic factor(s) is combined with protein of the blood, it will, of course, not diffuse from the dialysis sack. The factors determining whether or not such combination will occur are probably complex. There is, indeed, an interesting fact which indicates that the toxic factor(s) presumably does combine with protein. In the original experiments we found that, though it was always possible to extract toxic factor(s) from the defi-

brinated blood of the heat-killed or heat-injured animals, no toxic factor could ever be extracted if the blood was allowed to clot before dialysis was begun (see Fig. 4).

EVALUATION OF THE WORK UP TO THIS POINT

Our experiments have shown clearly that watery extracts of muscles and various other tissues contain a toxic substance or substances. Burned and heat-killed animals were found to contain this toxic factor(s) in large amounts. The substance (or substances) produces characteristic effects when injected into rats or mice, and the symptoms following such injections are strikingly similar to the symptoms exhibited by animals exposed to lethal temperatures in the hot room.

What is the nature of the toxic factor(s)? Our experiments showed (1) that it is readily dialysable; (2) that it is not, in general, destroyed by boiling; (3) that it is destroyed by vigorous ashing. In addition, numerous experiments showed that the toxic factor(s) is soluble in absolute methyl or ethyl alcohol. We were thus led to conclude that the toxic factor(s) in our experiments was a diffusible (nonprotein) substance and that it was organic rather than inorganic. However, the interesting paper of Pen, Campbell, and Manery (1944) led us to question whether our substance (or substances) was entirely organic. Pen, Campbell, and Manery (as also Macleod, 1907) found that the toxic substance which could be extracted from muscle was a salt of potassium. Actually, most of the results that we obtained could be explained on this basis. The fact that our preparations lost their potency following ashing was originally taken by us to mean that we could not be dealing with an inorganic salt. However, potassium chloride has a boiling-point between 1,400° and 1,500° C. (von Wartenberg and Albrecht [1921] give it as 1,417°). We did our ashing over a Meker burner, and the temperature of a Meker flame is 300°–400° higher than the boiling-point of potassium chloride.

Pen, Campbell, and Manery found that the intraperitoneal injection of potassium chloride into mice caused death. In their experiments, approximately 1 mg. of potassium chloride per gram of mouse was a lethal dose. (A slightly smaller lethal dose was noted by Emmens and Marks [1942].) We have confirmed this general finding; and, indeed, the mice or rats injected with potassium chloride show the symptoms we have described for rats killed by heat in the hot room or injected with our extracts.

At first sight, therefore, it seems probable that our results are to be explained by assuming that injured muscles or other tissues yield potassium and that this potassium is the toxic factor of our extracts. Such a conclusion would fit in with the views of those who maintain that an increase in blood potassium is an important factor in the production of shock (Zwemer and Scudder, 1938; Scudder, 1940; Cicardo, 1944).

Such a theory, however, could scarcely explain all the facts. In the first place, if potassium chloride is injected into the circulation, it generally causes an increase rather than a decrease in muscular strength. This, at any rate, is what Hoff, Winkler, and Smith (1941) found in their experiments on dogs. Eventually, when approximately ½ gm. of potassium chloride per kilogram of animal had been injected, the animal died of heart stoppage. Possibly more rapid injection of potassium chloride into the circulation might cause muscular weakness (cf. Brown and von Euler, 1938). Our experi-

ments on intravenous injection of tissue extracts into rabbits indicated that these extracts were far more toxic when so injected than when injected intraperitoneally. Also intravenous injection of the extracts caused a sharp fall in blood pressure; and injection of potassium chloride, as earlier authors have shown, does not have this effect. Presumably more work needs to be done. At the present time it is conceivable that intraperitoneal injection of potassium chloride does not act simply by introducing more potassium chloride into the circulation but has an indirect effect, perhaps causing the formation of toxic factor(s) or perhaps preventing the destruction of toxic factor(s) normally present (see below).

Second, the experiments on the blood of heat-killed rabbits and the blood of burned dogs are not reconcilable with the idea that potassium is the only toxic factor. Thus, we found that 15 ml. of blood from a burned dog was enough to kill 100 gm. of mice when injected intraperitoneally. But, according to Hoff, Winkler, and Smith (1941), the most potassium that can be present in the blood of a dog without causing heart stoppage is approximately 110 mg. per 100 ml., or 16.5 mg. in 15 ml. This, according to Pen, Campbell, and Manery, should only be enough to kill 16 gm. of mice. In other words, we found six times as much toxic factor(s) in the blood of a burned dog as could possibly be accounted for by the presence of potassium. The argument would be stronger if Frumin had been able to repeat our experiments on blood. The possible reasons for this failure have already been commented on.

Third, there is clear and direct evidence that watery and alcoholic extracts of all sorts of living tissue do actually contain toxic substances other than potassium. Such extracts can be shown to have marked effects on the protoplasm of the sea-urchin egg. This work will now be presented.

PROTOPLASMIC STUDIES

In the summer of 1944, experiments were begun on the effect of extracts of heated tissue on the protoplasm of sea-urchin eggs. The work was done at the Marine Biological Laboratory in Woods Hole, with the technical help of Bernice Goldis. Extracts were made of various types of living material, such as clams, sea anemones, lobster muscle, and squid muscle. The extracts were prepared by Wilson and Woodward. In the preparation of the extracts the living material was heated in several times its weight of distilled water for several hours. After filtration, the water extract was evaporated to dryness either on a steam bath or over a Bunsen burner, and the residue was then extracted with absolute methyl alcohol. This alcoholic extract was evaporated to dryness, and the residue was dissolved either in sea water or in distilled water.

An attempt was made to find out if the extracts had any effect on protoplasmic viscosity. A standard object for the measurement of protoplasmic viscosity is the egg of the sea-urchin, *Arbacia*, in which determinations are made by centrifuging the eggs (see Heilbrunn, 1928, 1943). A hand centrifuge is used, because it can be quickly started and stopped. The number of seconds of centrifugal treatment required to move the granules a given distance through the egg is taken as a measure of the protoplasmic viscosity. We used an Emerson hand centrifuge. If the handle of this centrifuge is turned once per second, it develops a force 9,896 times gravity. The eggs are placed in slender glass tubes fused at one end, and it is common practice to place a drop of isotonic sugar

solution in the bottom of these tubes so as to protect the eggs from injury against the glass. When normal eggs are centrifuged for 18 seconds, typically the granules are moved into two-thirds of the egg (somewhat longer centrifuging will move them into half of the egg). Throughout the summer the eggs do not vary greatly. We commonly use as an end-point the time required to move the granules into two-thirds of the egg, and this is taken as a viscosity value. Thus, with this technique, the normal value for the protoplasmic viscosity of the *Arbacia* egg is 18; during the summer, for various lots of eggs, it may vary from 15 to 20. Inasmuch as the absolute value for the protoplasmic viscosity is approximately 3 centipoises, the arbitrary centrifuge values can be translated into centipoises by dividing by 6.

Our 1944 experiments were not very satisfactory. We found, indeed, that, when *Arbacia* eggs were exposed to the extracts, the viscosity of the protoplasm rose, often very rapidly. Unfortunately, however, in most of our experiments we were not careful to control the tonicity of the extract solutions. If a solution is hypertonic, that in itself would tend to increase the protoplasmic viscosity. Figure 6 shows the type of data obtained in the 1944 experiments. The viscosity units are the arbitrary centrifuge units just described. The dotted line shows the effect of an extract of the sea anemone, *Metridium marginatum*. The increase in viscosity produced by this extract is very rapid and marked; for, within a half-minute, the viscosity rises to over seven times its original value. This particular extract was hypertonic, and it caused a 10.7 per cent decrease in the diameter of eggs left in it for half an hour. This represents a decrease in volume of the eggs of not quite 29 per cent. Strongly hypertonic solutions of pure salts, although they do cause viscosity increase, do not cause such a rapid and pronounced increase as that shown by the extract. Thus, when eggs were placed in equal parts of molar sodium chloride solution and sea water, the viscosity did not increase to twice its original value within an hour, although the solution in which the eggs were placed had an osmotic pressure nearly one and a half times that of ordinary sea water. When eggs were placed in

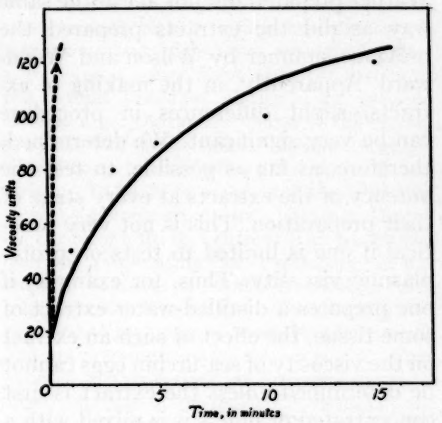

FIG. 6.—Effect of a *Metridium* extract on the protoplasmic viscosity of sea-urchin eggs.

equal parts of 2 molar sodium chloride and sea water (a mixture which has an osmotic pressure nearly three times that of sea water), the viscosity rose more rapidly and reached a value of 50 within half a minute. But even this viscosity rise is much less than that shown by the eggs in the extract. The extract was also diluted with an equal part of distilled water, and the eggs were placed in this diluted extract; the curve for the viscosity change is shown by the unbroken line in Figure 6. The diluted extract caused a marked increase in viscosity, and this effect almost certainly cannot be due to any hypertonicity. However, no direct determinations of the tonicity of the extract were made.

The results obtained during the summer of 1944 are admittedly not very satisfactory, although in a number of experiments viscosity curves like those of Figure 6 were obtained. Experiments were resumed during the summer of 1945. Mrs. Charlotte Warner helped with these later experiments, and she prepared the extracts, did freezing-point determinations, etc. The work began inauspiciously. The extracts that Mrs. Warner prepared did not act in the same way as did the extracts prepared the previous summer by Wilson and Woodward. Apparently, in the making of extracts, slight differences in procedure can be very significant. We determined, therefore, as far as possible, to test the potency of the extracts at every stage of their preparation. This is not very practical if one is limited to tests on protoplasmic viscosity. Thus, for example, if one prepares a distilled-water extract of some tissue, the effect of such an extract on the viscosity of sea-urchin eggs cannot be determined unless the extract is first concentrated or unless it is mixed with a relatively large volume of sea water (or unless salt be weighed out and added to it). For, if the eggs are placed in a very dilute extract, they immediately swell osmotically and if the increase in volume is great enough, this in itself causes a gelation of the protoplasm. Accordingly, we searched for some rapid and convenient method of testing the potency of extracts, no matter what their osmotic concentration.

When an *Arbacia* egg is broken so that the contents begin to flow out, the emerging protoplasm is immediately sealed off by a precipitation reaction which has been called the "surface precipitation reaction," or the "s.p.r." (see Heilbrunn, 1927, 1943). Normally, the s.p.r. occurs only if calcium is present in the surrounding medium. If a drop of oxalate solution is added to a drop of sea water containing eggs and the eggs are then crushed, the protoplasm flows out freely and no precipitation occurs. The reaction is comparable in many ways to a blood-clotting reaction; and, as in the case of blood clotting, thrombin or thrombin-like substances are formed which are able to produce an s.p.r. in the absence of calcium. Inasmuch as injured tissue can be a source of thrombin or thrombin-like substances, we decided to test our extracts in order to determine whether they would induce an s.p.r. in *Arbacia* eggs crushed in the absence of calcium.

The method did indeed prove useful. The following procedure was employed: A drop of tissue extract was mixed on a slide with a drop of 0.45-molar potassium oxalate solution; at the same time in an adjacent position on the slide a drop of sea water containing eggs was mixed with a drop of potassium oxalate solution. An interval of 1 minute was allowed to elapse in order to insure complete precipitation of calcium from the drops, and then the two drops were fused. As a result, the eggs in oxalated sea water came into contact with the oxalated extract. The preparation was covered with a cover slip, excess fluid was removed by absorbing it from the sides of the cover slip with filter paper, and the eggs were then broken by pressing down gently on the cover slip with the tip of a scalpel. In the presence of thrombin-like substances, an s.p.r. occurs even in the absence of free calcium.

During the course of the summer of 1945, extracts from a number of living tissues were tested for the presence of thrombin-like substances. Extracts were obtained from the following: whole sea anemones (*M. marginatum*); whole worms (*Nereis virens*); whole clams (*Venus mercenaria*); the mantles of squids (*Loligo peelei*); the muscles of lobsters (*Homarus*

americanus); the muscles of the toadfish (*Opsanus tau*); and the muscles of frogs (*Rana pipiens*). In preparing the extracts the tissue was cut up into small pieces or ground up in a meat-grinder. This was done after an early experiment with toadfish muscle, in which it appeared that cutting into small pieces or grinding resulted in a stronger extract than could be obtained if larger pieces were used. Also, in our first experiment on frog muscle, we found that, when the muscle was extracted in a dilute solution of either potassium chloride or calcium chloride, more potent extracts were obtained than when distilled water alone was used as the extraction medium. The amount of salt added was small.

The material in process of extraction was placed in an oven maintained at a temperature of 40°–50° C. (in one or two instances the temperature rose a few degrees higher). In every case, when tested in the manner above described, the extracts behaved as though they contained thrombin-like substances; that is to say, they were able to initiate an s.p.r. in *Arbacia* eggs broken in the absence of calcium. There were no exceptions, even though a large number of extracts was prepared. Thus, seventeen different extracts were made from frog muscles, and many of these extracts were treated in different ways, to give preparations of varying potency. The extracts do not all behave in identical fashion. For example, the extract from clams gave a better reaction if it was allowed to stay in contact with the eggs for 2 or 3 minutes before the eggs were crushed. This behavior was not typical.

So far the story is simple enough. But complexities began to appear when we tried to concentrate the extracts. Often enough, an extract which was effective in promoting a good s.p.r. in oxalated *Arbacia* eggs became completely ineffective following procedures designed to increase its strength. Thus, for example, an extract of toadfish muscle which was originally potent became completely impotent after it had been concentrated by boiling. A clam extract behaved in similar fashion, as is shown by the following record of an experiment:

85 grams of fresh clams were ground up in a meat grinder and extracted in 500 ml. of distilled water (plus a little toluene) for 4 hours at a temperature of 40–50° C. Samples of the extract were tested during the process of extraction, and such tests were made after 1, 2, 3 and 4 hours. Even after the first hour the extract produced an s.p.r. on oxalated *Arbacia* eggs. During the course of the extraction, the pH of the extract dropped slightly, from 6.60 to 6.54. (Such a pH drop is characteristic.) In order to avoid excessive heat, the extract was concentrated on a hot plate. During the period of heating, a current of air was passed over the evaporating dish containing the extract in order to facilitate evaporation at a relatively low temperature. The concentrated extract had a volume of 20 ml. Its pH was 5.67 and its freezing point was −1.962° C. Eggs exposed to this extract for 5 minutes showed a sharp increase in protoplasmic viscosity; the viscosity increased to more than double its original value. This could certainly not be accounted for by the slight hypertonicity of the extract. A drop of the concentrated extract was oxalated, and after a minute interval it was fused with a drop of oxalated *Arbacia* eggs. After another interval of several minutes, the eggs were crushed. The result was an intense s.p.r. A 5 ml. sample of the concentrated extract was then boiled in a flask with a reflux condenser for 5 hours. At the end of this time, the pH of the extract was 5.10. It was tested, as before, on oxalated *Arbacia* eggs, but it no longer showed any potency, and there was no s.p.r.

Obviously, boiling can destroy the potency of an extract; but this destruction is probably not the direct result of the heating. The following experiment is illustrative:

88 grams of fresh frog muscle were ground up in a meat grinder, and were then extracted with 176 ml. of distilled water plus 2 ml. of 0.8

molar $CaCl_2$ for 7 hours at a temperature of 40–56° C. The resultant extract, which was called F-2a, had a pH of 6.15 and a freezing point of −0.225° C. When F-2a was tested on oxalated *Arbacia* eggs in the usual way, the eggs gave a strong s.p.r. 160 ml. of F-2a were then concentrated to 20 ml. by heating on a hot plate over which a current of air from an electric fan was passed. (With our set-up the temperature did not rise above 70° C.) The concentrated extract is F-2b. It has a freezing point of −1.089° C. When F-2b was tested on oxalated *Arbacia* eggs, the eggs gave an s.p.r. reaction, but this was weaker than that produced by the much more dilute original extract (F-2a). 20 ml. of F-2b were concentrated to 10 ml., using hot plate and fan as before; the resultant extract is F-2c. It has a freezing point of −1.455° C. and a pH of 5.93. When F-2c was tested on oxalated *Arbacia* eggs, these showed essentially no s.p.r. The original extract (F-2a) was tested again; it gave a good reaction. We now took 10 ml. of F- 2a and diluted it with 70 ml. of absolute ethyl alcohol; this was evaporated to dryness with hot plate and fan, and the residue was dissolved in 2 ml. of distilled water. The undissolved portion of the residue was centrifuged off. The resultant extract was F-2d. When F-2d was tested on oxalated *Arbacia* eggs in the usual manner, the eggs showed an excellent s.p.r.

The above experiment shows clearly that when an extract is concentrated, even though the temperature at which concentration is effected is kept well below the boiling-point, a loss of potency may result. This loss of potency can scarcely be due to a volatilization of the active material, for such volatilization would also presumably have occurred in the preparation of extract F-2d, which remained potent. During the process of concentration of an extract, there is a loss of osmotically active solute. Thus, when extract F-2b was prepared from F-2a, although the volume was decreased to one-eighth the original volume and the concentration of solute should have increased eight times, the freezing-point lowering increased less than five times. Some of this discrepancy may be due to the fact that the extract may contain some relatively insoluble compounds; there may also be a slight effect due to the decrease in ionization or activity of salts at higher concentrations; but it is scarcely possible that either or both of these factors can account for the entire discrepancy. In all probability, as the extract is heated, protein comes out of solution, and this protein takes with it some dissolved substance or substances, including that portion of the extract which contains the thrombin-like substances that we are interested in. We can also assume that the addition of alcohol would tend to prevent the binding of the thrombin-like substance by the insoluble protein phase.

In later experiments we attempted to concentrate extracts and, at the same time, keep the thrombin-like substance free from the insoluble protein phase by bringing the extracts to a quick boil and then rapidly filtering off the precipitate. Once the precipitate was filtered off, we proceeded to concentrate the extract by prolonged boiling. Sometimes this method was effective, and sometimes it was not. Apparently, we are dealing with substances which can combine with proteins, and the success or failure of our efforts to obtain a potent extract depends largely on our ability to keep the active substance from becoming tied up in an insoluble protein phase. Sometimes this can be accomplished by a quick boiling and filtration, sometimes the addition of alcohol is helpful. Obviously, slight differences in the technique of preparing extracts may produce marked differences in their potency. We are thus in a position to understand why the extracts prepared in the summer of 1944 were almost all potent, whereas most of those prepared in the early part of the summer of 1945 were not potent in so far as their effect on protoplasmic viscosity was concerned.

Our improved knowledge with respect

to the preparation of potent extracts gave us courage to attempt again the study of the effects of extracts on protoplasmic viscosity. As was to be expected, the results obtained were somewhat variable; but we did clearly confirm the fact that extracts of injured tissue cause a great increase in protoplasmic viscosity. An early experiment on clam extract has already been mentioned. The following three experiments were concerned with extracts of frog muscle:

Extract F-9a was prepared by cutting up 30 grams of fresh frog muscle into small pieces. These were then extracted with 60 ml. distilled water plus 0.8 ml. 0.8 molar $CaCl_2$ for 7 hours at a temperature of 40–50° C. The extract was then filtered and the insoluble material discarded. F-9a has a pH of 5.80 and a freezing point of −0.260° C. 40 ml. of F-9a were heated to a boil, the precipitate that formed was filtered off and the filtrate was evaporated over a hot plate with the aid of an electric fan. Following evaporation to dryness, the residue was taken up in 4 ml. of distilled water. This is F-9b; its pH is 5.22, and its freezing point is −1.870° C. (The freezing point indicated no very great loss of solute, such as is apt to occur when extracts are concentrated.) *Arbacia* eggs were placed directly in F-9b, which was essentially isotonic. A centrifuge test after 6 minutes showed that the protoplasmic viscosity was well above 30; the viscosity of the control untreated eggs was 15. After 12 minutes exposure,, another test showed that the viscosity had not reached a value of 120. After 18 minutes, a third test showed the viscosity to be 100. It had therefore increased approximately 7 fold.

Extract F-12a was prepared from 70 grams of frog muscle cut into small pieces. The extraction was made with 140 ml. of distilled water plus 2 ml. 0.8 molar $CaCl_2$. The muscle stayed in this solution for 8 hours at a temperature of 40–50° C. F-12a had a pH of 6.94 and a freezing point of −0.559° C. 80 ml. of F-12a were brought to a boil; the precipitate that formed was filtered off, and the filtrate evaporated to dryness with hot plate and fan. The residue was dissolved in 50 ml. absolute methyl alcohol. This alcoholic solution was filtered, and the filtrate evaporated to dryness. The residue was then dissolved in 8 ml. of distilled water. This is F-12b; its pH is 5.25 and its freezing point −1.512° C. (The freezing point is not as low as it would have been if the methyl alcohol extraction had been omitted, for much salt was not taken up by the methyl alcohol.) Eggs were placed in F-12b and the viscosity of the protoplasm was then tested. A solution of the osmotic strength of F-12b ordinarily causes a marked decrease in viscosity [see Heilbrunn, 1928, pp. 211–13]. However, F-12b caused a very sharp viscosity increase. A test made after 12 minutes exposure showed the viscosity to be far above 30; another test made after 16 minutes showed the viscosity to be well over 120. Thus F-12b caused at least a seven fold increase in viscosity.

Extract F-10a was obtained from 56 grams of frog muscle cut into small pieces and soaked in 112 ml. of distilled water plus 1.3 ml. 0.8 molar $CaCl_2$ for 7 hours at a temperature of 40–50° C. The pH of the filtered extract was 5.96; its freezing point was −0.282° C. To 25 ml. of F-10a, 200 ml. of absolute alcohol was added, and the whole solution was evaporated to dryness. The residue was dissolved in 3 ml. of distilled water and filtered. This is F-10d; its pH is 5.75 and its freezing point (approximately) −1.728° C. Eggs were placed in F-10d and the viscosity of the protoplasm was tested after 5 minutes exposure and again after 30 minutes exposure. Both tests showed that the viscosity had not approached a value of 30 and thus apparently there was no marked increase above the normal.

These experiments show that extracts of frog muscle can have a powerful effect on the viscosity of the protoplasm of sea-urchin eggs. The effect is not due to the pH of the solutions; at any rate, ordinary mineral acids at pH 5 have no marked effect on the protoplasmic viscosity of *Arbacia* eggs, even after the eggs have been exposed to such solutions for an hour. It is to be noted that two of the extracts had a very marked effect on the protoplasmic viscosity; the third extract had little or no effect. And yet all three extracts, when tested on oxalated *Arbacia* eggs, gave a good s.p.r. Indeed, extract F-10d, which had little or no effect on protoplasmic viscosity, gave an exceptionally strong s.p.r. The most plaus-

ible explanation seems to be that, although the extracts contain thrombin-like substances, these are sometimes able to enter the cells rapidly and sometimes not. The rate of penetration of a thrombin-like substance presumably depends on its molecular size, but it may also depend on the possible presence in the extracts of substances which can alter the permeability of the plasma membrane so as to make it more or less permeable to the thrombin-like substances.

The season for *Arbacia* eggs in Woods Hole normally ends in early September. Late in August we ran across a new development in our viscosity measurements. We had been studying an extract prepared from lobster muscle, as follows:

50 grams of fresh lobster muscle were cut into small pieces and extracted with 100 ml. of distilled water for 8 hours at 40–50° C. The extract was then filtered. It is L-1a and it has a pH of 7.02 and a freezing point of −0.646° C. 90 ml. of L-1a were brought to a boil. A precipitate formed, and this was filtered off after cooling. The filtrate was evaporated to dryness, and the residue dissolved in 25 ml. absolute methyl alcohol. Following filtration, the filtrate was again evaporated to dryness and the residue dissolved in 25 ml. distilled water. This is L-1b. Its pH is 5.30 and its freezing point −0.842° C. 14 ml. of L-1b were concentrated by evaporation with the aid of a hot plate and fan to a volume of 7 ml. This is L-1c; it has a pH of 5.40 and a freezing point of −1.646° C. Thus it is slightly hypotonic.

Arbacia eggs were placed in extract L-1c, and 5 minutes later a viscosity test was made. The eggs were given 30 seconds of centrifugal treatment at the usual speed. Normal eggs treated in this fashion have the heavier granules moved into approximately half of the egg. All the heavy granules move, with the exception of those in a single thin layer closely applied to the outer membrane of the egg cell. The average diameter of the granules in this thin cortical layer is 0.79 μ, according to Moser (1939). But now, in the eggs exposed to L-1c, instead of a thin, single-layered cortex after centrifugal treatment, there was a cortex which consisted of many layers of granules—a cortex which was at least 10 μ thick. In this test, made after 5 minutes' exposure, there is thus evidence of a gelation limited to the periphery of the egg. After 14 minutes' exposure to the extract, the eggs were centrifuged again and for the same length of time. Following this centrifugal treatment, the eggs showed no movement of granules at all, so that the gelation originally confined to the cortex had moved through the entire egg.

Somewhat similar results were obtained with extracts from frog muscle:

Extract F-11a was prepared by extracting 50 grams of minced frog muscle with 100 ml. distilled water plus 1 ml. 0.8 molar $CaCl_2$ for 7 hours at 40–50° C. The filtered extract has a pH of 6.02 and a freezing point of −0.200° C. 50 ml. of F-11a was brought to a boil and the precipitate that formed was filtered off. The filtrate was evaporated to dryness with hot plate and fan, and the residue was dissolved in 25 ml. absolute methyl alcohol. The extract was then filtered and evaporated to dryness; the residue was dissolved in 5 ml. of distilled water and filtered. At this stage the pH was 5.55 and the freezing point −0.790° C. 4 ml. of this extract was concentrated with hot plate and fan to a volume of 2 ml. This is F-11d. Although no freezing point determination was made, it is obviously slightly hypotonic. Eggs exposed for 6 minutes to F-11d were centrifuged in the usual way for 30 seconds. They showed a cortex which was somewhat thicker than normal. Another test, made after 30 minutes exposure, showed a cortex at least 10 or 15 microns thick.

Extracts F-13b and F-14b were prepared in essentially the same way as F-11d. F-13b had a pH of 5.85 and a freezing point of −1.517° C. F-14b had a pH of 5.75 and a freezing point of −1.597° C. Eggs exposed to F-13b and centrifuged at various times showed a cortex which became increasingly thick. The eggs were centrifuged in the usual way for 30 seconds following exposures of 5, 11 and 20 minutes. By 5 minutes, the cortex was already thick;

after 20 minutes, the cortex was at least 20 microns thick (the diameter of the whole egg is about 75 microns). Eggs exposed to F-14b showed a similar behavior. They were centrifuged (for 30 seconds) after exposures of 5, 10 and 30 minutes. Increased thickness of the cortex was very evident after 5 minutes; after 30 minutes the cortex was very thick, but the entire egg was not yet gelated.

The experiments just reported show clearly that extracts from lobster muscle and from frog muscle may contain a substance (or substances) which has a rather peculiar effect on the viscosity of the protoplasm of the sea-urchin egg. In the past experience of the senior author with various types of substances which cause increase in protoplasmic viscosity, most of them were found to act almost simultaneously on all parts of the protoplasm. In other words, if a viscosity-increasing substance enters the cell through its plasma membrane, it ordinarily diffuses very quickly through the mass of the protoplasm. On the other hand, a thrombin-like substance, one capable of producing a surface precipitation membrane, might continually be forming precipitates which barred its rapid progress through the protoplasm. It must not be thought, however, that the phenomenon of a cortical gelation is unique. When *Arbacia* eggs are fertilized, there is an immediate and intense increase in the viscosity of the cortical region of the cell. Also, exposure to ultraviolet radiation may cause a cortical gelation; this was reported many years ago by Heilbrunn and Young (1930).

In the course of our studies on the effect of tissue extracts on protoplasm, we stumbled onto a fact which may prove to have rather wide significance. The first observation was made on the fourth of our extracts from squid muscle. The report of the experiment follows.

37 grams of fresh squid muscle were cut into small pieces and extracted with 74 ml. of distilled water for 7 hours at 40–50° C. The extract was then filtered. It is S-4a and its pH is 6.75, its freezing point −0.390° C. Tested on oxalated *Arbacia* eggs, it gives a rather good s.p.r. To 35 ml. of S-4a 280 ml. of absolute methyl alcohol was added, the precipitate that formed was filtered off immediately, and the filtrate evaporated to dryness in an evaporating dish with hot plate and fan. The residue was dissolved in 5 ml. distilled water and filtered. This is S-4c. Its pH is 5.72 and its freezing point −1.660° C. After dissolving the residue in the evaporating dish and pouring this off as S-4c, another 5 ml. of distilled water was added to the evaporating dish and stirred around. The resultant extract is S-4c′. Its pH is 5.67 and its freezing point −0.219° C. When extracts S-4c and S-4c′ were tested on oxalated *Arbacia* eggs in the usual way, it was found that S-4c′ gave a sharper and more pronounced reaction than S-4c.

The above experiment was interpreted in the following way: The original extract contained thrombin-like substance and also some inhibiting substance. When, after the addition of alcohol, the extract was evaporated to dryness and the residue dissolved in a small amount of water, the new extract contained, in addition to the active substance(s), a relatively greater amount of the inhibiting substance. This would be easily understandable if the inhibiting substance were more readily soluble in water than the active substance. The experiment with squid muscle was repeated, perhaps more strikingly, with sea anemones. Owing to the fact that these animals are hollow and contain large amounts of sea water, they were extracted in a small volume of distilled water.

25 ml. of distilled water was added to about 130 grams of sea-anemones (*Metridium marginatum*), and the anemones were extracted in this small amount of water for 8 hours at 40–50° C. The filtered extract is SA-1a; its pH is 7.49 and its freezing point −0.821° C. To 40 ml. of SA-1a, 280 ml. of absolute ethyl alcohol was added, the precipitate that formed was filtered off and the extract evaporated to dryness. The residue was dissolved in 13 ml. of distilled

water and filtered. This is SA-1b; its pH is 5.45 and its freezing point −1.815° C. The undissolved residue was extracted a second time with 13 ml. of distilled water, and in this way extract SA-1c was obtained; its pH is 5.87 and its freezing point −0.076° C. A third extraction of the undissolved residue was made with 10 ml. of distilled water and the resultant extract is SA-1d. Its pH is 6.60 and its freezing point 0.000 °C., that is to say, its freezing point is essentially that of distilled water. When the original extract, SA-1a, was tested on oxalated *Arbacia* eggs, it gave a good s.p.r. On the other hand, SA-1b, when tested in similar fashion, gave a very weak reaction, hardly any reaction at all. And yet, extracts SA-1c and SA-1d gave clear surface precipitation reactions, especially if only a small number of eggs was present when the test was made.

There thus is a strong reason to assume the existence of an inhibiting substance. What is the nature of the substance? It must, of course, be present in the original water extracts, but it seems to gain strongly in inhibiting power after it is concentrated. The suspicion arose that the inhibitor was simply sodium chloride. Accordingly, experiments were tried to test this hypothesis. Sodium chloride was added to potent extracts from sea anemones, from lobster muscle, and from frog muscle, and the effect on the s.p.r.-producing power of the extract was noted. The procedure was as follows: On a microscope slide a drop of extract was mixed with a drop of 0.53 molar sodium chloride (or potassium chloride) solution and a drop of sea water containing eggs. All drops were of standard size (0.03 ml.). After 1 minute, a drop of 0.45 molar potassium oxalate solution was added. Another minute was allowed to elapse, in order to allow time for the thorough precipitation of calcium. Then the eggs were tested for s.p.r. In general, the addition of sodium weakened or prevented the s.p.r., the addition of potassium did not have this effect.

There is thus evidence that sodium chloride can exert an inhibiting effect on the s.p.r. when the reaction takes place in oxalated cells in the presence of a thrombin-like substance. Unfortunately, the evidence is not so clear as we should like to have it. Further experiments are needed, but these were scarcely possible in the summer of 1945, for the sodium effect was noted only at the very end of the *Arbacia* egg season. In judging the validity of the evidence, it should be remembered that s.p.r. tests are not rigidly quantitative. The reaction of the eggs depends to some extent on the force with which they are broken, and this could not be accurately controlled. In the experiments described, the sodium chloride exerted its inhibiting action when it was added to both eggs and extract. It is not certain whether the presence of eggs is necessary for the weakening or destruction of the thrombin-like substance of the extract. Critical experiments could be planned. For the present, we are inclined to believe that the sodium exerts its inhibiting or destroying effect on the thrombin-like substance of the tissue extracts by acting in co-operation with some substance contained in or given off by the eggs. But this is merely a guess.

On the whole, it seems reasonably clear that sodium does actually favor the destruction of thrombin-like substances produced by injured tissues. If this is true, then it is easy to understand why sodium has been successfully used as a therapeutic agent following shock (see Rosenthal, 1943; Prinzmetal, Hechter, Margoles, and Feigen, 1943; Fox, 1944). This aspect of the subject will be considered more fully in the section that follows.

FINAL DISCUSSION

The work on simple cells has helped to clarify our earlier experiments on complicated higher animals. (These earlier

experiments were discussed in another section of this paper.) It is clear from the experiments on *Arbacia* eggs that many diverse types of living tissues on injury produce thrombin-like substances which can have a marked effect on protoplasm. The preparation of concentrated extracts of these substances is beset with difficulty, for they appear to have a tendency to combine with protein. Accordingly, when an extract is concentrated by heat, the thrombin-like substance originally present in the extract may combine with an insoluble protein precipitate and be filtered off. It is thus possible to understand why variable results were obtained in the preparations of potent extracts. Slight variations in procedure may in one instance permit the active substance to stay in solution, or they may in another instance cause the substance to become part of an insoluble precipitate. Moreover, as noted previously, combination with protein may prevent toxic factor(s) in the blood from passing through a dialysis sack.

The active thrombin-like substances from all sorts of living organisms and tissues can cause clotting of the protoplasm of broken or crushed sea-urchin eggs in the absence of calcium. If the substances enter the egg cells, they cause a gelation of the protoplasm there. Such a gelation would, of course, produce marked effects on various types of sensitive tissues, and it might account for some of the phenomena observed in shocked animals.

That thrombin or thrombin-like substances may play a part in shock is strongly indicated by the work of those authors who have found that both thrombin and trypsin cause shock if injected into rabbits or dogs (Tagnon, 1945; Rocha e Silva, 1940). Trypsin, it will be remembered, acts like thrombin in causing the coagulation of oxalated shed blood (Eagle and Harris, 1937). Strangely enough, when thrombin and trypsin are injected intravenously, they cause a delay in the coagulation time of the blood. This was shown by Tagnon (1945) and, for trypsin, even earlier, by Davis (1911). Decreased coagulability of the blood is a characteristic of various types of shock, including anaphylactic shock (see, e.g., Biedl and Kraus, 1909; Krueger and Schultz, 1925). Interestingly enough, the oxalated blood from shocked animals clots more readily outside the body than does normal oxalated blood (see Irish, Hamilton, Haist, and Jacques 1945).

The idea that thrombin might play a part in burn-shock seems to have been common enough in the 1880's (see, e.g., von Lesser, 1880; Foà, 1881). Although it is true that small amounts of thrombin injected into the blood stream cause a delay in blood-clotting time, larger amounts cause clotting. Sometimes, following burns, thrombi appear in the circulation. Schjerning (1884), in his survey of the burn literature, lists eight cases in which such thrombi were described and states that thrombi are more common after burn wounds than after ordinary wounds. Von Lesser points out that the pathological picture observed in blood, intestine, and kidney following lethal burns is essentially identical ("gleicht fast absolut") with that which may be observed following the injection of thrombin. Foà drew blood from a dog, allowed it to clot, and then injected fluid squeezed out from the clot back into the dog. The animal showed symptoms similar to those observable after burns.

When mammalian tissues are injured, doubtless both potassium and thrombin are released into the blood stream. There can scarcely be any question of this. Potassium alone cannot be responsible for all the symptoms shown by shocked ani-

mals, for the potassium content of the blood does not rise sufficiently after shock to account for all these symptoms. This is rather clearly shown by Harkins (1942) in his review of the literature on burn-shock (see pp. 55, 56; see also Manery and Solandt, 1943). Nevertheless, it is important to note that either potassium alone or thrombin alone can cause a shocklike condition. If it is true, as it certainly is, that injured tissues release both potassium and thrombin, then we have an interpretation of the nature of the toxic factor(s) in terms of substances known to be present following injury.

A particularly interesting point is the fact that potassium seems to prevent the destruction of thrombin-like substances, or, rather, an increase in the potassium-sodium ratio tends to prevent this destruction. Quite conceivably, thrombin or thrombin-like substances are circulating in the blood of normal animals at all times. The intraperitoneal injection of potassium might prevent the destruction of such substances, and this is one possible explanation of the toxic action of potassium following its intraperitoneal injection.

If sodium tends to destroy shock-producing thrombin, then, clearly, it is advantageous to supply shocked animals with excess sodium. Here, then, is a rational explanation of the mechanism of sodium therapy in shock. In a recent review, one of the leading authorities on burns and burn-shock states: "The use of sodium therapy in burns is one of the chief points of interest at the present time" (Harkins, 1945).

Logically, the administration of sodium should be of benefit to men or animals exposed to excessive heat of the environment. For, indeed, the injuries caused by "heat stroke" may not be very different from the injuries caused by the local application of excessive heat. Both following heat stroke and following burns, a state of shock may ensue. It is not surprising, therefore, that for many years inhabitants of hot climates and workers in industries in which there is exposure to excessive heat have found some measure of relief by eating sodium chloride. This has generally been interpreted as being completely explained on the assumption that the chloride ingested replaces the chloride lost through the sweat. However, in view of the previous discussion, is it not just as probable that the sodium of the sodium chloride is really the effective agent in counteracting some of the effects of the excessive heat?

But, aside from these matters of practical clinical importance, it is a fact of broad general significance that injured tissues of all types produce marked effects on uninjured cells. This point of view was emphasized by the senior author many years ago, long before it was known that thrombin could produce a condition of shock. The results of this paper and also the work of Tagnon (1945) lend strong support to the following statement, made first in 1937:

> It is apparently a general attribute of protoplasm that when it is injured or excited it produces substances which have an exciting effect on resting protoplasm with which they come into contact. This fact might perhaps be brought into relation with the colloid chemical theory of excitation. . . . . According to this theory, the essential change produced in protoplasm as a result of excitation (or injury) is a protoplasmic clotting analogous to blood clotting and rather closely related to it. If this theory is correct, it is easy to understand why excitation or injury should produce substances capable of excitation effects in resting protoplasm. When blood clots, there is always a production of very potent substances which cause clotting in other samples of blood. It is possible that protoplasmic clotting also produces substances of the same type. In the case of sea-urchins, recent experiments have shown that injury of various tissues produces substances

which cause (*a*) contraction of smooth muscle, and (*b*) clotting of the perivisceral fluid or blood. In these animals, therefore, it is true that injury substances affect both blood clotting and protoplasm. In vertebrates, moreover, there is an abundance of evidence that substances produced as a result of cell injury have an effect on blood clotting, and that, on the other hand, substances produced by blood clotting may influence excitation. Thus . . . . when blood serum . . . . is injected into the blood stream or perfused through blood vessels, it causes a marked contraction of the smooth muscles in the walls of the vessel [Heilbrunn, 1937, p. 458].

SUMMARY

The following summary is not a complete outline of the paper, but it covers the main points.

1. The heat-death symptoms of rats are described, and comparisons are made of the heat-death temperatures of rats, mice, rabbits, guinea pigs, and cats. Rats in a hot room die at temperatures of 45° C. or even lower.

2. If the hind legs of rats are exposed to temperatures of 45° C., the animals die in about 2½ hours. This death does not occur if the legs are tightly ligatured. Guinea pigs are not so sensitive to partial exposure to heat.

3. When the legs of rats are heat-killed, the muscles die before the nerves. Also in frog nerve-muscle preparations the muscle is more sensitive to heat than is the nerve.

4. Extracts of heat-killed frog muscle hasten the heat death of other frog muscles. Similar results can also be obtained with mammalian muscles.

5. Water extracts of muscle, when injected intraperitoneally into a rat, cause death, with symptoms similar to those which occur when the animal is exposed to excessive heat. Assays indicate that hot-water extracts have a higher potency than cold-water extracts. Extracts from muscles of heat-killed animals are also very potent.

6. The toxic substance(s) of muscle extracts is not species specific, and an extract from one of the several mammals studied acts on rats or mice.

7. Extracts from all sorts of organs and tissues produce effects similar to the muscle extracts. Assays indicated that extracts from muscles were more potent than those from other tissues or organs.

8. The toxic substance(s) of these extracts is dialysable and is soluble in absolute methyl or ethyl alcohol.

9. In several experiments the blood of heat-killed rabbits was found to contain some toxic substance(s) which caused heat-death symptoms when injected into rats. The toxic substance(s) was obtained by dialyzing the blood.

10. In three experiments, the blood of scalded dogs was found to contain toxic substance(s) which killed rats in the same way that the muscle extract did. This toxic substance(s) was obtained by dialyzing the blood. Later experiments (by another worker) failed to confirm this result, but such lack of confirmation is not regarded as destroying the significance of the successful experiments. As will be noted later, slight differences in technique are very important in the preparation of extracts.

11. All the extracts discussed thus far certainly contained potassium, and some of their effects may well have been due to this factor. Nevertheless, the results do not appear to be entirely explicable on the basis of any potassium theory.

12. Extracts from a wide variety of heat-killed organisms and tissues (sea anemones, worms, clams, squids, lobster muscle, fish muscle, and frog muscle) all contain a substance which has an effect on the clotting of oxalated protoplasm. In other words, the extracts cause an s.p.r. in oxalated sea-urchin eggs. This reaction offers a convenient

method of testing the potency of an extract at different stages in its preparation.

13. Although the water extracts of many types of heat-injured tissue are effective in producing the protoplasm-clotting reaction, attempts to concentrate the potent (thrombin-like) substance(s) were not always successful.

14. Often the extracts lost their potency when heated, and this loss is apparently due to a tendency for the active substance(s) to combine with an insoluble protein phase. Slight differences in the technique of preparing extracts may have a marked influence on their potency.

15. The extracts from various types of heat-killed tissues were found to cause a sharp increase in the protoplasmic viscosity of the intact sea-urchin egg.

16. In some cases the extracts produced a rather peculiar increase in protoplasmic viscosity, for the viscosity increase was, for a time, confined to the cortical region of the cell.

17. Thus the studies with sea-urchin eggs indicate clearly that many diverse types of living material, on heat injury, yield watery extracts containing thrombin-like substances. In every case the thrombin-like substances of the extracts cause an s.p.r. in oxalated sea-urchin eggs. In many cases, also, the extracts caused the protoplasm to gel within the cells.

18. The thrombin-like activity of the extracts from heated tissues and organisms seems to be lost in the presence of sufficient concentrations of sodium. A high sodium-potassium ratio would thus tend to rob the extracts of the power to clot protoplasm.

19. These findings are to be correlated with the known fact that thrombin can cause shock when injected into the circulation. It is clear that thrombin and thrombin-like substances can have powerful effects on cells into which the thrombin is able to penetrate.

20. Inasmuch as injured tissues in mammals are known to yield both thrombin and potassium, it is easy to understand why injury of one sort or another can cause shock or shocklike symptoms, for either of these substances is effective by itself. It is possible that potassium acts indirectly by preserving thrombin from destruction.

21. In the light of our results, there is an obvious reason for the success of sodium therapy in burn-shock. Moreover, inasmuch as localized heat, which causes burns, and environmental heat, which causes heat stroke, both can produce shock, it would seem logical to suppose that, if sodium chloride is helpful in burn-shock because of its sodium, the same compound would be helpful in over-all exposure to heat because of its sodium rather than because of its chloride.

22. The significance of the results is considered in relation to the colloid chemical theory of excitation.

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STUDIES ON THE NUTRITION OF COLORLESS EUGLENOID FLAGELLATES. II. GROWTH OF ASTASIA IN AN INORGANIC MEDIUM[1,2]

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THE presence or absence of chlorophyll is usually regarded as determining whether an organism is capable of autotrophic nutrition. Those species possessing chlorophyll are regarded as "autotrophs," while the colorless forms are considered capable of growth only when organic compounds are furnished. The only generally recognized exceptions to this rule are found in the bacteria, in which certain species, even though they do not possess chlorophyll, are nevertheless capable of growth in inorganic media (chemosynthetic or chemoautotrophic nutrition). Other groups of microörganisms are usually regarded as following the concepts of "animal" and "plant" nutrition, but they have not been studied so extensively as the bacteria. In this regard the flagellates offer interesting experimental material, for in this group of the Protozoa some species are green, while closely related forms are colorless.

The nutritional requirements of a number of flagellates have been determined. Most of the green species investigated have been found capable of autotrophic nutrition (Pringsheim, 1912; Mainx, 1928; Dusi, 1933*a*, *b*; Loefer, 1934; Hall, 1938, 1939; Hall and Schoenborn, 1938, 1939; Osterud, 1939). On the other hand, the simplest media found satisfactory for growth of the colorless flagellates (with one exception) have contained an organic carbon source in addition to the various inorganic salts. Media of this nature have been reported as satisfactory for *Polytoma uvella* and *Polytomella caeca* by Pringsheim (1937); for these two species plus *Polytoma obtusum*, *Polytomella caudatum*, and *Polytomella ocellatum* by Lwoff and Dusi (1938); and for *Astasia* sp. by Schoenborn (1940*a*). The exception noted above is *Chilomonas paramecium*. According to Mast and Pace (1933), this colorless species is capable of chemoautotrophic nutrition, provided that silicon is present and the CO_2 concentration of the air is increased. Other workers, however, have reported more complex nutritional requirements for different strains of *C. paramecium* (Pringsheim, 1935; Hall and Loefer, 1936; Lwoff and Dusi, 1938; Ondratschek, 1940).

The author's interest in the possibility of chemoautotrophic nutrition in Protista other than bacteria was aroused when *Astasia*, one of the colorless euglenoid flagellates, was successfully cultured in a medium containing acetate as the only organic material (Schoenborn, 1940*a*). Upon removal of acetate from the medium, growth continued. This led to the work described below.

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MATERIAL AND METHODS

The organism studied, *Astasia* sp., was isolated, bacteria-free, by Dr. T. L. Jahn and is the same strain that has been previously studied by the author (Schoenborn, 1936, 1940*a*, *b*, *c*).

Two media were utilized in most of the work described below. Medium A contained $(NH_4)_2HPO_4$, 1.0 gm.; $MgSO_4 \cdot 7H_2O$, 0.2 gm.; KH_2PO_4, 0.2 gm.; KCl, 0.2 gm.; $FeCl_3 \cdot 6H_2O$, 0.0025 gm.; distilled water, 1.0 liter. Medium B contained the same salts plus 1.0 gm. of sodium acetate per liter. All chemicals used were of analytical grade, and the water was at least double distilled.

One of the most convenient methods for determining whether an organism is capable of continuous growth under certain conditions is the serial-transfer technique. This method was used in obtaining many of the results to be described. Medium for the first transfer was prepared and adjusted to a suitable pH with HCl (Schoenborn, 1936); pH determinations were made either colorimetrically or with a glass electrode. This medium was then measured into tubes or flasks, sterilized for 20 minutes at 115° C., and inoculated with a strain of *Astasia*. After a suitable period of incubation, one of these first-transfer cultures was used to inoculate second-transfer culture vessels, similarly prepared. The third transfer was inoculated from a second-transfer culture, etc.

When culture tubes were used, at least ten were prepared for each transfer. Two of these tubes were used for initial and final pH determinations; three were fixed immediately after inoculation (by the addition of 0.5 cc. of fixative) for the determination of the initial density of population; and after the incubation period three more tubes were fixed to determine the final population density. One or two samples (1 cc. each) were removed from each of the fixed tubes, placed in a Sedgwick-Rafter counting chamber, and the number of organisms counted by means of a Whipple micrometer (Hall, Johnson, and Loefer, 1935). The counts obtained from these samples were averaged to give the initial and final population densities. When flasks were used as culture vessels, the population densities were obtained in one of two ways: (1) by counts on 1-cc. samples aseptically withdrawn from the flasks; or (2) by calculation from the population density of the culture used to inoculate the flask or of the cultures inoculated from the flask. Growth is expressed as the ratio of final to initial population density (x/x_0) and hence refers to growth of the population (Tables 1, 2, 5).

All cultures were kept at room temperature, and, since light has a harmful effect on the growth of *Astasia* in simple media (Schoenborn, 1940*a*, *c*), the cultures were grown in darkness.

Chemical cleanliness of glassware is of the utmost importance in nutritional studies of microörganisms. While soap was used in the early experiments to clean all glassware, in the later series (V–XII) only acid bichromate cleaning solution was employed; or, if soap was used, it was followed by the acid cleaning solution. After thorough rinsing, the culture vessels and pipettes were plugged with bleached cotton and hot-air sterilized. Any toxic effects resulting from the use of bichromate cleaning solution (Richards, 1935) are important in studies of this nature only when no growth is observed. Positive results warrant no conclusion regarding the effect of bichromate, but they do indicate that the organism is capable of growth in a given solution.

EXPERIMENTAL RESULTS

The simplest medium previously used for continuous growth of *Astasia* (Schoen-

born, 1940*a*) consisted of various inorganic salts and an organic carbon source, sodium acetate (medium B). Series I in the present investigation was the initial attempt to ascertain whether removal of sodium acetate from this medium resulted in the cessation of growth. Series II was the control series, in which the medium contained acetate. Both tubes (16 × 150 mm.) and flasks (125-cc. Erlenmeyer) were used, the former receiving 9 cc. of medium and a 1-cc. inoculum, while the latter received four times these amounts. A flask culture of any particular transfer was used to inoculate the tubes and flasks of the next transfer. All transfers of Series I and II had an initial pH of 6.7. In this experiment, as in all later ones, the final pH did not vary more than 0.2 from the initial reading. Incubation periods for the transfers varied from 21 to 37 days. The first transfers of these two series, inoculated with a strain which had been grown in the acetate-containing solution for eleven transfers covering a period of over 6 months, received an insignificant amount of complex organic material commonly used in stock cultures (approximately 9×10^{-16} gm. per cubic centimeter of peptone). During the various transfers of Series I, the acetate concentration was progressively reduced by dilution; by the twenty-fourth transfer it had been reduced to 1×10^{-27} gm. per cubic centimeter. From the results (Table 1) it is seen that growth continued in both media for the duration of the experiment, twenty-four successive transfers covering a period of over 19 months. The final population densities obtained in the acetate-containing medium were usually greater than those obtained in the purely inorganic solution. The x/x_0 values in the two media are not greatly different; but this is to be expected, for the ratio of inoculum to medium was 1 : 9 in both cases; and later results (Sers. III–IV) show that the incubation periods were generally of sufficient length to allow the population to reach the maximal stationary phase of population growth.

Whereas the results just described indicate that *Astasia* is capable of continuous growth in an inorganic solution, previous preliminary studies with the same medium had led to the opposite conclusion (Schoenborn, 1940*a*). Several differences existed between these two sets of experiments, one being the source of flagellates introduced into the inorganic solution. In the earlier investigation, a strain grown in peptone medium furnished organisms for the first transfer, while Series I of the current report was inoculated from a strain in acetate-containing medium B. If the latter strain had undergone adaptation or selection, it might more easily be transferred to a purely inorganic solution than a strain grown on such complex substances as peptones. In order to test this possibility, two series in the inorganic solution were started. These two series were identical, except that one was seeded from a strain in peptone medium while the other was inoculated with a strain cultured in medium B. Growth continued in both series for eight transfers, at which time the experiment was stopped. Later work confirmed these results. Hence, it is possible to establish a strain in an inorganic medium directly from a strain in peptone medium, and the reasons for the previous failure to obtain growth of *Astasia* in an inorganic solution remain unknown.

EVIDENCE OF CHEMOAUTOTROPHIC NUTRITION FROM GROWTH CURVES

The results obtained in these initial experiments suggested that *Astasia* is capable of continuous growth in a medium containing no added organic carbon source. But, even though no or-

TABLE 1

GROWTH IN A PURELY INORGANIC MEDIUM (SER. I) AND IN THE SAME MEDIUM PLUS SODIUM ACETATE (SER. II)

Population densities are expressed as the number of organisms per cubic centimeter; population growth as the ratio of final to initial density of population.

Series	Transfer	Initial Density of Population	Final Density of Population		Population Growth	
			Tube Cultures	Flask Cultures	Tube Cultures	Flask Cultures
I.....	1	133	1,370	2,500	10.3	18.8
	2	250	1,237	2,130	4.9	8.5
	3	213	1,077	1,400	5.1	6.6
	4	140	2,273	3,570	16.2	25.5
	5	357	1,473	2,070	4.1	5.8
	6	207	1,697	3,000	8.2	14.5
	7	300	1,860	*	6.2	*
	8	127	1,457	1,580	11.5	12.4
	9	158	1,267	1,470	8.0	9.3
	10	147	917	770	6.2	5.2
	11	77	473	730	6.1	9.5
	12	73	820	1,350	11.2	18.5
	13	135	1,200	1,370	8.9	10.1
	14	137	570	1,100	4.2	8.0
	15	110	1,173	1,900	10.7	17.3
	16	190	837	1,100	4.4	5.8
	17	110	670	1,070	6.1	9.7
	18	107	627	1,030	5.9	9.6
	19	103	617	800	6.0	7.8
	20	80	703	800	8.8	10.0
	21	80	667	1,130	8.3	14.1
	22	113	950	1,570	8.4	13.9
	23	157	743	800	4.7	5.1
	24	80	855	.....	10.7	.....
II.....	1	133	1,650	2,670	12.4	20.1
	2	267	1,830	2,100	6.9	7.9
	3	210	1,450	2,230	6.9	10.6
	4	223	2,517	4,000	11.3	17.9
	5	400	1,940	2,870	4.9	7.2
	6	287	1,960	2,700	6.8	9.4
	7	270	3,233	2,370	12.0	8.8
	8	237	2,063	2,530	8.7	10.7
	9	253	1,243	1,300	4.9	5.1
	10	130	1,387	2,470	10.7	19.0
	11	247	1,617	2,670	6.5	10.8
	12	267	2,303	2,730	8.6	10.2
	13	273	3,863	3,970	14.2	14.5
	14	397	1,637	1,500	4.1	3.8
	15	150	1,220	1,930	8.1	12.9
	16	193	2,227	2,630	11.5	13.6
	17	263	1,583	1,670	6.0	6.3
	18	167	1,860	2,570	11.1	15.4
	19	257	3,743	4,670	14.6	18.2
	20	467	2,353	6,870	5.0	14.7
	21	687	3,323	3,600	4.8	5.2
	22	360	2,627	3,300	7.3	9.2
	23	330	2,023	2,630	6.1	8.0
	24	263	1,535	.....	5.8	.....

* Flasks contaminated; next transfer inoculated from tube cultures.

ganic substance was added to the medium, it seemed possible that contamination of the cultures with traces of organic compounds might have been responsible for the growth obtained. If so, growth curves might furnish evidence on this point. That is, if traces of necessary organic substances were present in the medium, growth might occur for the first few days following inoculation of fresh medium but would soon cease when the essential organic materials were consumed. Such results have been reported by Lwoff and Dusi (1938) for various colorless flagellates grown in the presence of essential growth factors. Experiments to test this possibility are described below.

Series III and IV.—Population growth curves in the inorganic medium were traced for both flask and tube cultures. Five 125-cc. Erlenmeyer flasks, each with 45 cc. of medium and a 5-cc. inoculum from the nineteenth transfer of Series I, were used in Series III. Initial pH was 6.7. By using flasks it was possible to follow the population increase in the same vessels. Counts were made on one or two samples (1 cc. each) withdrawn aseptically from each of three flasks at intervals. The results (Fig. 1) show that the population continued to increase for about 31 days. In an experiment extending over this length of time, it is possible that evaporation might account for some of the increase in population density. This possibility was investigated in Series IV, in which tubes containing 9 cc. of medium and a 1-cc. inoculum (same source as Ser. III) were used. Initial pH was 6.7. The population density at indicated intervals was determined by averaging counts of two samples from each of three tubes. In making corrections for

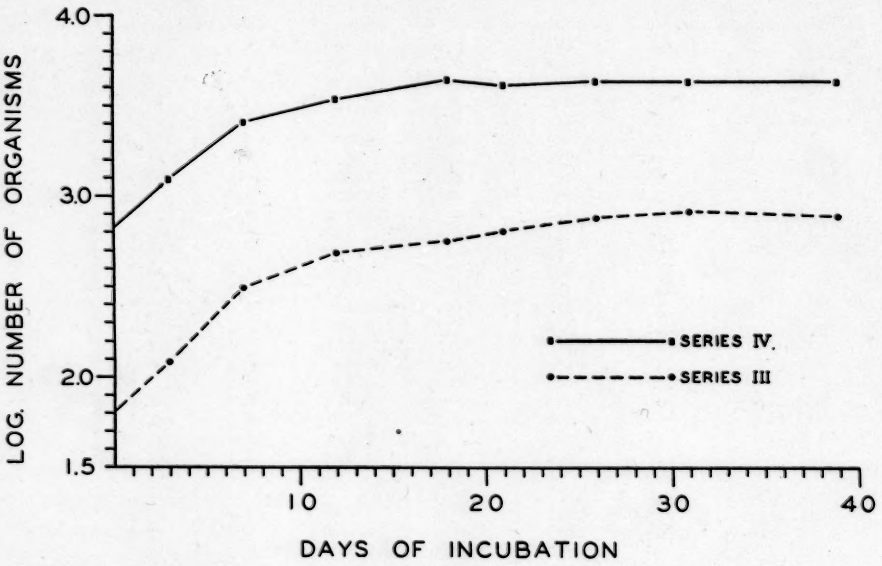

FIG. 1.—Population increase in inorganic medium. Flask cultures used in Series III; each point on curve represents the log number of organisms per cubic centimeter. Tube cultures utilized in Series IV; each point represents the log number of organisms per tube.

evaporation, the volumes of the sampled cultures were measured, averaged, and the number of organisms *per tube* calculated. The results (Fig. 1) showed that in tube cultures the population continued to increase for about 18 days and then remained in the maximal stationary phase for some time. This may indicate that some of the reported increase in the flask cultures of Series III may have been an error traceable to evaporation; but, since there are other differences between flask and tube cultures—such as the rate of gas exchange—it cannot be concluded that evaporation is the sole factor responsible for the apparently longer period of growth in flasks. However, both Series III and Series IV show that growth is not limited to the first few days after inoculation but extends over a rather long period of time, thus indicating that growth in the inorganic medium is not dependent upon a small and limited supply of organic substances which can be rapidly metabolized.

CONTAMINATION OF MEDIUM WITH ORGANIC COMPOUNDS IN COTTON PLUGS

Further possible sources of organic contaminants were considered. If traces of organic substances were present in the medium, they must have gained access from the cotton plugs used to stopper the culture vessels, as impurities in the salts or in the water employed, from the air, or perhaps from the autoclave. The possible contribution of nutritive materials from cotton plugs was tested in Series V and VI.

Series V and VI.—Pyrex distilling flasks (50-cc.) were used as culture vessels. In both series the side arm was plugged with cotton; since the side arm slants downward, there is little chance of cotton fibers falling into the medium or of the medium coming into contact with the cotton during autoclaving. The mouth of each flask was closed by an inverted vial in Series V, while in Series VI cotton plugs were used. Growth in the inorganic medium was followed for five serial transfers in these two series, both of which were seeded initially from a twentieth-transfer flask culture of Series I. In each transfer the initial pH was 6.7. Incubation periods were 21–29 days. The final population densities in successive transfers (Table 2) indicate that the absence of cotton plugs in the mouths of the Series V flasks did not inhibit growth of *Astasia*. It can be concluded, therefore, that any organic substances contributed to the medium by the cotton plugs were not responsible for growth of *Astasia* in the inorganic solution employed.

CONTAMINATION OF MEDIUM WITH ORGANIC COMPOUNDS DURING STERILIZATION

Unfortunately, the strain of *Astasia* used in the above work was lost in transit. Hence new strains had to be established in the inorganic solution for later work on this problem. The strain used to inoculate the two series next described had been grown in the inorganic medium for four transfers covering a period of over 3 months, during which time the calculated peptone concentration was reduced, by dilution, to 2.19×10^{-8} gm. per cubic centimeter.

Series VII and VIII.—These series were designed to determine whether any organic materials entered the medium during autoclaving and were responsible for growth of *Astasia* in the inorganic solution. Eight 125-cc. Florence flasks, each with 18 cc. of medium, served as culture vessels. Four of these flasks, used for Series VII, were sterilized in an autoclave which had been used for various organic media and the remaining flasks, for Series VIII, in a pressure cooker which had not been used prior to this ex-

periment. Thus there should be no possibility of organic materials' entering the medium of Series VIII during sterilization. Each flask received a 2-cc. inoculum. The initial pH was 6.9 in the first and 6.1 in the remaining two transfers. In the third transfer the population densities were determined after varying periods of time. Results (Fig. 2) show that any organic materials which may enter the medium during autoclaving are not essential for growth in the inorganic solution.

Series IX and X.—In order to determine whether the inorganic solution was receiving essential organic materials from the air, cultures were aerated with air which was free of organic carbon compounds. This was accomplished by utilizing the method described by Ter Meulen and Heslinga (1932) for carbon determinations. Air (with 5 per cent CO_2) was slowly passed through a quartz tube containing a mixture of MnO_2 and PbO_2, heated to 300° C. This treatment should oxidize any organic material in the air to CO_2. After being cooled, the air was passed through the experimental cultures. The culture vessels were eight 250-cc. aeration flasks, each containing 100 cc. of the inorganic medium and a 4-cc. inoculum from the culture described above. For Series IX, six of the flasks were connected in series to the quartz tube, and thus the cultures were exposed to air free of organic carbon compounds. The calculated peptone concentration in these flasks was 4.3×10^{-10} gm. per cubic centimeter. After an incubation period of 17 days, during which time the initial population increased 9.8 times, one of these flasks was used to inoculate the series described below.

TABLE 2

COMPARISON OF GROWTH IN VESSELS NOT STOPPERED (SER. V) AND STOPPERED (SER. VI) WITH COTTON PLUGS

Population densities are expressed as the number of flagellates per cubic centimeter; population growth as the ratio of final to initial density of population.

Transfer	Series V			Series VI		
	Initial Population Density	Final Population Density	Population Growth	Initial Population Density	Final Population Density	Population Growth
1	96	536	5.6	87	593	6.8
2	45	996	22.1	55	1,242	22.6
3	103	1,047	10.2	117	1,348	11.5
4	99	1,021	10.3	129	1,036	8.0
5	102	650	6.4	95	820	8.6

CONTAMINATION OF MEDIUM WITH ORGANIC COMPOUNDS IN AIR

The next factor to be studied was the possibility that organic carbon compounds might enter the medium from the air. A new strain of *Astasia* in the inorganic medium was again established. This was accomplished by seeding a centrifuge tube containing sterile inorganic medium with 1 cc. of a peptone stock culture. After thorough mixing and centrifugation, 1 cc. of material from the bottom of the tube was transferred to a second tube. In this way the organisms were carried through 6 tubes, the last of which served to inoculate culture flasks of inorganic medium. The calcu-

The other two flasks, used for Series X, were merely exposed to laboratory air. Initial pH was 6.3 in Series IX and 6.7 in Series X. The distilled water employed in making the medium for these two series was carefully prepared. It was four times distilled; the second distillation was made in the presence of potassium persulphate and the third with potassium permanganate.

The initial population density for Series IX and X was 80 organisms per cubic centimeter. After an incubation period of 18 days, the final population densities were 415 in Series IX and 360 in Series X. Thus growth in cultures exposed to "pure" air was fully as good as in cultures exposed to ordinary air. As a further check, the final population densities of the six flasks exposed to "pure" air were compared. If the oxidation of organic compounds had not been completed in the quartz combustion tube and assuming that these compounds were water soluble, it might be expected that a greater percentage of the carbon compounds would be dissolved in flask 1 (the one nearest the quartz tube) than in the other flasks of the series. If these compounds were necessary for growth of *Astasia*, one should observe a larger final population in flask 1, and perhaps progressively smaller populations in the remaining flasks. Such results were not observed. Instead, the first two flasks had slightly smaller populations (357 and 385 organisms per cubic centimeter) than the remaining flasks (493, 415, 440, 403, respectively).

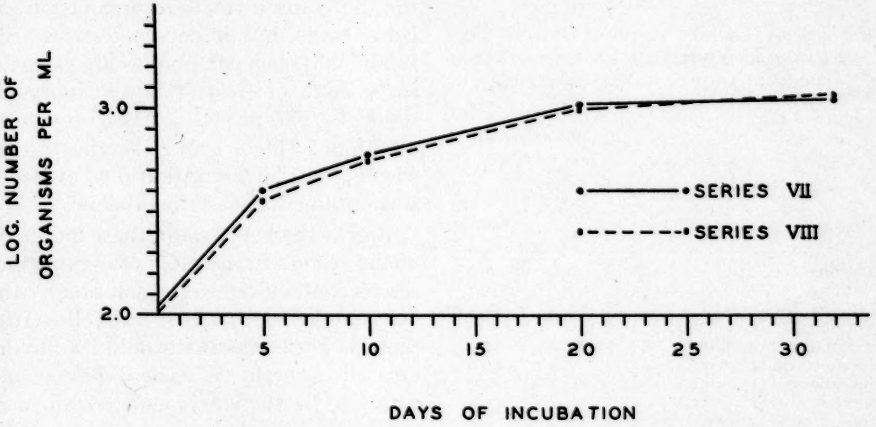

FIG. 2.—Population increase (expressed as log number of organisms per cubic centimeter) in inorganic medium sterilized in a "used" autoclave (Ser. VII) and in a "new" autoclave (Ser. VIII).

CONTAMINATION OF MEDIUM WITH ORGANIC COMPOUNDS IN WATER AND SALTS

The remaining two possibilities whereby the inorganic medium might become enriched with organic nutritives are the chemicals and the water. Assuming that the organic materials are supplied by the chemicals, an inorganic medium differing in composition from the one ordinarily used might give interesting results. Accordingly, growth was followed for 7 transfers in a medium composed of the following salts per liter of water: NH_4Cl, 0.497 gm.; $MgSO_4$, 0.048 gm.; $K_2HPO_4 \cdot 3H_2O$, 0.209 gm.; $FeCl_3 \cdot 6H_2O$, 0.0025

gm. The first transfer in this medium was inoculated from the seventeenth transfer of Series I. Initial pH was 6.6–6.7, and the growth periods ranged from 21 to 37 days. Growth in this medium was comparable to that observed in the solution usually employed.

It also seemed advisable to use inorganic salts from different sources. In the medium just described and in that employed for Series I–VI, Mallinckrodt chemicals were used. A combination of Merck and Baker and Adamson chemicals was used in Series VII–XII. Growth occurred in all cases, but this may indicate only that both groups of salts contained traces of organic substances. Consequently, carbon determinations were made on samples of the inorganic compounds employed in the later series.

TABLE 3

RESULTS OF CARBON DETERMINATIONS WITH NOTHING AND WITH KNOWN AMOUNTS OF SUCCINIC ACID IN COMBUSTION TUBE

SAMPLE	CO_2 COLLECTED (IN MG.)	
	Theoretical	Observed
Nothing	0	0.6
Nothing	0	0.4
Nothing	0	0.4
Nothing	0	0.3
Succinic acid (20.0 mg.)	29.8	29.5
Succinic acid (18.0 mg.)	26.8	27.0
Succinic acid (17.6 mg.)	26.2	26.5
Succinic acid (11.7 mg.)	17.5	17.4
Succinic acid (5.6 mg.)	8.4	8.8
Succinic acid (3.8 mg.)	5.7	5.7

These determinations were made according to the Ter Meulen and Heslinga (1932) method; precautions were taken, as recommended, for sulphur-, nitrogen-, and halogen-containing substances. The amount of carbon in the sample can be calculated from the quantity of CO_2 collected. To test the accuracy of the apparatus, four "blank" runs were made, as well as six determinations on known amounts of succinic acid. Results are given in Table 3. The largest error observed in any of these determinations was 0.6 mg. Determinations were then made on the four main constituents of the inorganic solution. Results are shown in Table 4. According to these findings, the four salts tested would collectively contribute approximately 0.67 mg. of carbon to each liter of medium.

These results indicate that the medium did contain traces of organic carbon compounds, but it does not necessarily follow that such compounds are essential for growth of *Astasia* in the inorganic solution. The growth curves previously mentioned (Figs. 1 and 2) indicate that such is not the case. Additional evidence was obtained as described below.

If growth of *Astasia* in these inorganic media results from traces of organic materials contributed to the solution by the salts employed, then it should follow that dilution of the medium would lead to decreased growth. Whether that would prove to be the case was ascertained in Series IX, which has already been described. Besides the six flasks containing full-strength medium, other aeration flasks were prepared in which the medium was diluted 1 : 1, 1 : 3, and 1 : 9; all dilutions were made with the specially prepared distilled water previously described. Initial pH ranged from 5.8 in the 1 : 9 dilution up to 6.3 for the full-strength medium. "Pure" air was bubbled through all flasks; those with dilute medium were connected in series to the last flask having full-strength medium. The initial density of population was 80 organisms per cubic centimeter; the final population densities were 415 in full-strength medium, 417 in the 1 : 1 dilution, 385 in the 1 : 3 dilution, and 430 in the 1 : 9 dilution. Thus dilution of

the medium, within the limits specified, does not lead to decreased growth. This indicates that any organic materials contributed to the medium by the salts are not responsible for growth of *Astasia* in the inorganic solution. And, since the distilled water used in this series was carefully prepared, it seems unlikely that organic nutritives responsible for growth could come from that source.

TABLE 4

RESULTS OF CARBON DETERMINATIONS ON CONSTITUENTS OF THE INORGANIC MEDIUM

Sample (In Gm.)	CO_2 Collected (In Mg.)	Calculated Carbon per Gram of Substance (In Mg.)	Calculated Carbon Contributed to 1 Liter of Medium (In Mg.)
$(NH_4)_2HPO_4$ (1.0386)	4.2	1.1	.....
$(NH_4)_2HPO_4$ (0.5210)	0.7	0.4	.....
$(NH_4)_2HPO_4$ (0.5271)	0.4	0.2	.....
$(NH_4)_2HPO_4$ (0.9041)	0.8	0.2	.....
Average	.....	0.48	0.48
KH_2PO_4 (0.8135)	0.4	0.1	.....
KH_2PO_4 (0.5525)	0.3	0.2	.....
Average	.....	0.15	0.03
$MgSO_4 \cdot 7H_2O$ (0.6229)	0.9	0.4	.....
$MgSO_4 \cdot 7H_2O$ (0.3335)	0.6	0.5	.....
Average	.....	0.45	0.09
KCl (1.3861)	1.2	0.2	.....
KCl (0.3867)	0.8	0.6	.....
KCl (0.2220)	−0.5	0.0	.....
KCl (0.4050)	0.9	0.6	.....
Average	.....	0.35	0.07
Total	.....	.....	0.67

EFFECT OF CARBON DIOXIDE ON GROWTH

All the results described above would indicate that *Astasia* is capable of growth in a purely inorganic solution. Under such conditions the carbon requirements of the organism must be furnished by CO_2 in the air. Therefore, interesting results might be obtained if the CO_2 concentration was increased over that ordinarily found in air. Preliminary studies were made as described below.

Series XI and XII.—The strain used to inoculate Series XI was obtained in the same way as the strain employed to seed Series IX and X. Organisms from a peptone stock culture were carried through a number of washings in sterile inorganic medium and were then used to inoculate the experimental cultures. In this way the concentration of organic material was rapidly reduced; the cultures of Series XI had a calculated peptone concentration of 4.3×10^{-10} gm. per cubic centimeter. Three aeration flasks were prepared, each containing 75 cc. of medium and a 1.5-cc. inoculum. Five per cent CO_2 in air was bubbled

through one flask at a rate of about 10 bubbles per minute; ordinary air was bubbled through the second flask at a corresponding rate; and the third flask was not aerated. The culture subjected to 5 per cent CO_2 had an initial pH of 6.3, and the other two flasks 6.7. The initial count was 230 organisms per cubic centimeter; the growth period lasted 17 days. From the results (Table 5), it is seen that the final population was largest in the flask aerated with 5 per cent CO_2, somewhat lower in the nonaerated flask, and smallest in the flask aerated with ordinary air. Series XII was a repetition of Series XI in all respects except that a new strain was prepared (but in the same manner), and the experimental cultures therefore had the higher initial count of 372 organisms per cubic centimeter. Results in Series XII (Table 5) were similar to those obtained in Series XI.

TABLE 5

GROWTH IN INORGANIC MEDIUM AERATED UNDER DIFFERENT CONDITIONS

The final population densities are expressed as the number of organisms per cubic centimeter; population growth as the ratio of final to initial density of population.

Series	Gas Bubbled through Culture	Final Population Density	Population Growth
XI.....	5 per cent CO_2 in air	2,250	9.8
	Air	1,055	4.6
	Nothing	1,960	8.5
XII.....	5 per cent CO_2 in air	3,775	10.1
	Air	2,020	5.4
	Nothing	2,570	6.9

As summarized by Miller (1938), the rate of photosynthesis in green plants can be increased by increasing the CO_2 content of the atmosphere. If capable of chemoautotrophic nutrition, *Astasia* must also assimilate CO_2, and the results of the above series indicate that here also an increase in CO_2 concentration resulted in a more rapid synthetic rate. The differences in growth observed between the air-bubbled cultures and the nonbubbled cultures might also be explained by differences in CO_2 concentration. If bubbling air through the cultures removed the CO_2 produced during respiration more rapidly than it was removed by diffusion in the nonbubbled cultures, these latter cultures may have a slightly higher CO_2 content than the air-bubbled flasks and therefore be capable of supporting better growth.

DISCUSSION

The results described above support the idea that *Astasia* sp., one of the colorless euglenoid flagellates, is capable of chemoautotrophic (or chemosynthetic) nutrition. The inorganic solution permitting growth contained $(NH_4)_2HPO_4$, KH_2PO_4, $MgSO_4$, KCl, and $FeCl_3$. It cannot be concluded, however, that only the elements present in these salts are necessary for growth of *Astasia;* for, although the salts employed were of analytical grade, they still contain traces of other elements which may later prove to be indispensable. But in a simple solution containing these inorganic salts (plus "trace" elements as contaminants?), *Astasia* is evidently capable of synthesizing all necessary protoplasmic constituents. In these syntheses it must be assumed that CO_2 serves as the sole carbon source, that inorganic nitrogen is utilized, and that the necessary energy for metabolic processes is obtained through the oxidation of some inorganic constituent of the medium. No evidence can be obtained regarding CO_2 as the sole carbon source by removal of CO_2 from the medium; for, even in a peptone medium, *Astasia* sp. does not grow in the absence of CO_2 (Rahn, 1941). It has been shown above that a CO_2 concentration

greater than that found in ordinary air leads to increased growth; but the crucial evidence as to whether CO_2 is utilized as the sole carbon source will have to come from biochemical investigations. Such investigations would also undoubtedly provide valuable information regarding nitrogen utilization and the material which serves as the energy source for growth in the inorganic solution.

Before studies of a biochemical nature are undertaken, it seems desirable to attempt to increase growth in these simple solutions. This might be accomplished by determining essential elements and their optimal concentrations or by studying the effects of various growth stimulants. It is also possible that other species of colorless flagellates will be found which grow more rapidly in inorganic media than does *Astasia* and would thus be more suitable for biochemical studies.

. The extent of chemoautotrophic nutrition among the colorless flagellates is as yet unknown. Mast and Pace (1933) reported a cryptomonad, *C. paramecium*, as capable of chemoautotrophic nutrition; but the strains of *C. paramecium* studied by Pringsheim (1935), Hall and Loefer (1936), Lwoff and Dusi (1938) and Ondratschek (1940) were found to have more complex nutritional requirements. In the Euglenida, *Astasia* sp., as studied above, is the only reported chemoautotrophic species. *Euglena gracilis*, when cultured in darkness, has been found incapable of growth in an inorganic solution unless acetate is present (Schoenborn, 1942). A number of colorless phytomonads have been cultured in inorganic solutions plus sodium acetate; but few reports record any attempt to culture the organisms in purely inorganic media. It might not be surprising, therefore, to find other colorless flagellates capable of chemosynthetic nutrition.

SUMMARY

1. *Astasia* sp., a colorless euglenoid flagellate, has been grown in pure culture in a purely inorganic medium.

2. Growth curves indicate that this growth does not result from traces of organic substances in the medium.

3. It has been shown that any organic materials which may gain access to the medium during autoclaving, from cotton plugs, as impurities in the salts employed, or from the air are not responsible for the growth observed.

4. Preliminary studies indicate that a CO_2 concentration greater than that in air permits better growth in the inorganic solution.

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NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

GENERAL BIOLOGY AND PHILOSOPHY OF ORGANISM[1]
BY RALPH STAYNER LILLIE

Reviewed by MICHAEL F. GUYER
University of Wisconsin

THIS book embodies the matured concepts of a distinguished physiologist who has long thought and written about the philosophical implications arising from our knowledge of living organisms and of nature in general. Accepting mechanism as obvious, he finds no ultimate explanations in it. As a realist he is trying to look beyond mechanism.

Basing his discussion, as far as possible, on tested scientific knowledge, he directs his attention to the metaphysical aspects of the factors which underlie the coming of novelty and of change in the natural world, that is, of that diversification which must be the basis of all evolution, whether inorganic or organic. Obviously, nothing can originate in nature without some form of change. If materials, conditions, relations, and modes of action remained wholly constant, then natural phenomena could only persist as unchanging entities. While, unquestionably, natural processes do tend to repeat themselves, nevertheless, in a long view of nature it is evident that vast changes have occurred. Greater and greater complexities have arisen, and more and more kinds of materials and, notably, of living organisms have appeared. Lillie maintains that "spontaneous," unpredictable activities abound in nature for which no known physically identifiable causes or conditions adequately account. It is for the appearance of these novelties and their shaping into complex, unified, self-maintaining wholes that he seeks an answer.

He appeals to Heisenberg's principle of indeterminacy as sufficient to free natural events from absolute invariance; and this possible lapse from fixity once granted, then a certain measure of spontaneity becomes not only possible but inevitable. But the impressive thing, according to him, is the fact that these internally developed new factors, wholly or largely independent of the past conditions of the object itself or of its environment, do not continue to exist as casual or random elements but come to assert themselves in a unified, effective manner. The explanation of this unifying, adaptive influence, he believes, can be found only in psychic factors, ordinarily ignored or overlooked by the scientist. Under appropriate circumstances this psychic force may guide vital activities in a manner largely independent of environmental conditions.

Why psychic? His justification for using this concept is that it is only within one's own conscious mental life that one can find a parallel to this integrative procedure. He points out that the inner

[1] Chicago: University of Chicago Press, 1945. Pp. 215. $3.00.

aspects of life, felt as direct experience, are just as actual as the material substances composing the body and must therefore be reckoned with, if one is to gain a truthful concept of the whole being. Such nonphysical attributes Lillie designates as "psychical," since they are interpretable only in terms of what we ordinarily designate as "psychic processes." That we commonly regard the physical and the psychical activities of nature as wholly different means only, according to him, that, instead of regarding them as together forming the natural psychophysical unity which they actually do form, we are accustomed to represent these two kinds of activity intellectually in different terms.

To have a complete explanation of the actual happenings in nature, he maintains, one must recognize this directive force, psychic in character, along with the physical and measurable. It is not something wholly apart from the physical, however, but an attribute of it, the two together constituting a psychophysical unity. They are not separable or capable of independent existence. Grounded in the same foundational reality, they merely represent different aspects of that reality.

It is this psychic aspect that renders aim and directiveness possible in the otherwise purely material. Without it there would be no selection among qualities; everything would, in accordance with Newton's second law of thermodynamics, tend more and more toward uniformity and consequent stalemate.

Merely to recognize the obvious part which purpose plays in our own human activities is to admit that planning or "aim" is possible in nature; and, since there is no sharp demarcation between the conscious mental activities of man and the higher animals and the probably unconscious adaptive behavior of lower organisms, including embryos or even plants, some measure of the psychic, he argues, must also be conceded to them. All alike manifest the existence of a general, purposeful, integrative property that is not merely the chance outcome of the interaction of their chemical constituents. Aim, plan, and selection are present throughout; recognition of teleological factors is inescapable. And these are of the nature of psychism. As to how the psychic factor changes the physical condition, he admits that there is no available scientific solution at present; that such psychic factors exist we can only accept as a fact of universal human experience.

The reviewer suspects that, while biologists will admit his conclusions about the inadequacy of our present knowledge of physics and chemistry to explain vital phenomena and evolution in general—and, above all, psychic phenomena—most of them will fight shy of postulating any correlative influence pervading all nature which they are willing to label "psychic." They will probably prefer for the present to represent the unknown in nature just by a question mark (?). To get the full force of Lillie's argument, however, one must read the book.

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